THE EFFECTS OF ADRENOCORTICAL HORMONES ON ALBUMIN METABOLISM STUDIED WITH ALBUMIN-I^{131} [1]

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Previous studies of serum albumin turnover, employing albumin-I^{131} as a tracer, have revealed a narrow range of values averaging about 200 mg. per Kg. per day in normal male adults and in patients with congestive heart failure (1, 2). In other disease states, abnormalities in the rate of synthesis or of degradation or of both have been observed. In patients with marked proteinuria, the amount of albumin undergoing metabolic degradation was found to be diminished significantly in most cases, whereas the rate of synthesis remained at normal levels (2). In contrast, the abnormally high rate of albumin catabolism in thyrotoxicosis was observed to be compensated by an augmented synthesis of albumin (3).

The major effect of the adrenocortical hormones on protein metabolism has been interpreted as an increased rate of catabolism (4), a decreased rate of synthesis (5) or a combination of both effects (6, 7, 8). It appeared to be of interest to study the influence of these hormones on serum albumin metabolism since methods have been developed (3) that readily permit distinction between catabolic and antianabolic effects on serum albumin even under nonsteady state conditions. The present study is concerned with alterations in the rates of synthesis and degradation of serum albumin following treatment of rabbits and human subjects with steroid hormones.

METHODS

Eight healthy female rabbits were kept in metabolism cages and fed a standard Rockland rabbit diet, *ad lib.* Lugol's solution was added to the drinking water daily. During the period of cortisone administration, each rabbit received 40 mg. of streptomycin intramuscularly and 75,000 units of penicillin intramuscularly every other day to prevent infection. At the onset of cortisone administration, 5 to 8 mEq. of potassium chloride was added to the drinking water, of which the animals consumed a quantity equivalent to 2 to 3 mEq. of potassium chloride daily.

The distribution and metabolism of albumin were studied by means of I^{131} labeled rabbit serum albumin. Following the intravenous administration of albumin-I^{131}, assays of I^{131} in plasma, stool and urine were made for a control period of 10 to 14 days and continued through an experimental period of another 13 to 19 days, during which time cortisone acetate was given subcutaneously in doses of 3 mg. per Kg. per day for the first 5 days and 1.5 mg. per Kg. per day thereafter. A second dose of albumin-I^{131} was administered at the end of this period and observations were continued for another 9 to 16 days, during which time the animals continued to receive cortisone. Injections of albumin-I^{131} were made into an ear vein and 0.3 to 0.5 ml. of heparinized venous blood was obtained from the opposite ear 6 minutes and 10 minutes later and at daily intervals for one week, and every second to third day thereafter. The six minute serum sample was separated by paper electrophoresis in a veronal buffer, pH 8.6 ($\mu = 0.05$). A single radioactive peak was observed migrating with the albumin band (Figure 1). Paper electrophoretograms of rabbit serum, following the administration of 250 μc. of sodium iodide, were stained and washed in the same fashion as that shown in Figure 1. No significant evidence of radioactivity was obtained. Twenty-four hour urine and stool collections were obtained daily except in occasional instances when pooled weekend collections were made.

One hundred and fifty μc. of iodinated pooled human serum albumin,[2] with a specific activity not greater than 50 μc. per mg., was administered intravenously to five male patients at the New York Veterans Administration Hospital, and observations on plasma and urinary I^{131} were made for control periods of 14 to 19 days, following which adrenocortical hormones were administered and observations continued for another 13 to 20 days. A second injection of albumin-I^{131} was then made and observations were continued for 14 to 19 days, during which time hormone administration was continued in all subjects except Patient T. H. Patients J. O., J. A. and T. H. received prednisone 10 mg. three times daily by mouth. Patient A. V. received hydrocortisone 150 mg.

[1] This material was presented in part at the meeting of the Eastern Section of the American Federation for Clinical Research on December 6, 1957.

[2] Salt poor pooled human serum albumin, obtained from Cutter Laboratories, California, was used in these studies. Paper electrophoresis of this material revealed a single sharp band both before and after iodination. Iodination was performed as previously described (1).

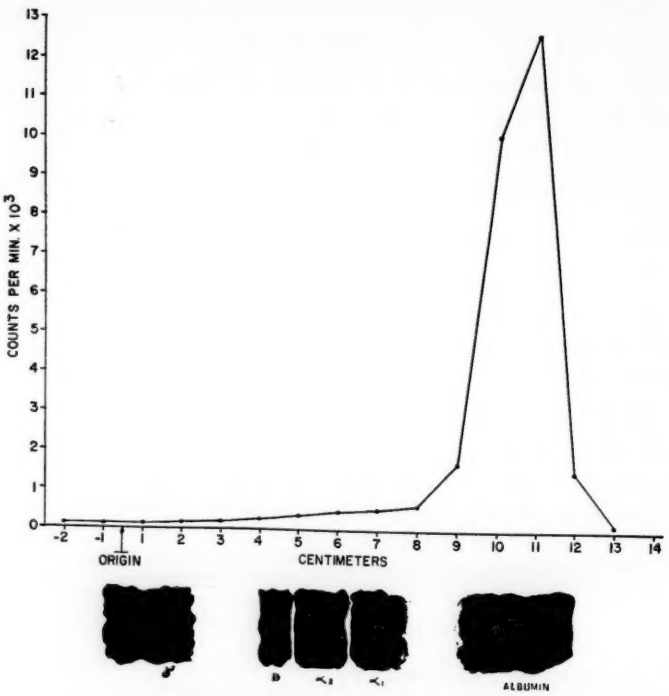

FIG. 1. PAPER RADIOELECTROPHORETOGRAM OF A SIX MINUTE SAMPLE OF RABBIT PLASMA

daily orally and Patient T. W. received prednisone 10 mg. orally three times daily for 10 days and then twice daily. On the thirteenth day of drug administration, Patient T. H. developed gastrointestinal complaints and prednisone was discontinued on the day of the second albumin-I^{131} injection. The patients received 10 drops of Lugol's solution twice daily and were maintained on a low salt diet without a potassium supplement.

Plasma samples were obtained from the arm opposite to the side of injection at 15, 18 and 21 minutes and at almost daily intervals thereafter. Daily urine collections were obtained except for an occasional pooled week-end sample.

The rabbit albumin was prepared by zone electrophoresis on a Geon 426 block in a veronal buffer, pH 8.6 ($\mu = 0.075$). Five ml. of pooled rabbit serum was added to the block as a narrow band. The apparatus was placed in a cold room at 4 to 7° C. and a potential of 600 volts was applied for 24 hours. The albumin band was located by placing the edge of a strip of Whatman 3 MM paper along the longitudinal axis of the block. When the liquid phase reached a height of 5 mm., the paper was removed, dried at 110° C. for 30 minutes and stained with bromphenol blue. The albumin zone on the resin block was divided into four bands and the two middle bands were eluted with saline. The eluate was dialyzed against distilled water, lyophilized to a small volume, and rerun on a Geon 426 block. The final preparation ran as a single component. The middle fraction of the albumin was eluted as before and lyophilized to a dry solid after dialysis. The albumin was iodinated as described previously (1) and contained approximately 1 iodine atom per protein molecule with a specific activity not greater than 50 μc. per mg. of protein.

Plasma, urine and stool samples were assayed for radioactivity in a well-type scintillation counter with a sensitivity of 9.7×10^8 counts per minute per μc. I^{131} above a background of approximately 170 counts per minute. Total serum protein was determined by the Kjeldahl method and serum albumin concentration was determined by the method of Kingsley (9) at 4 to 7 day intervals throughout the period of study. Control and experimental sera were analyzed by free and zone electrophoresis at pH 8.6. No significant differences in the alpha globulin fractions were observed. Urine was tested for protein by precipitation with cold trichloracetic acid and blood sugar was determined by the method of Somogyi (10).

Plasma volume was determined from the space of distribution of albumin-I^{131} at 6 and 10 minutes in the

animals and at 15 and 18 minutes in the patients. Total exchangeable albumin and the extravascular and intravascular distribution of albumin were determined according to methods described previously (1). The rate of albumin metabolism during the control period was determined by two methods, as (*A*) the product of total exchangeable albumin and the rate constant of decline in plasma radioactivity after distribution equilibrium, and (*B*) as the product of the "metabolic clearance" of albumin-I^{131} and the serum albumin concentration. These methods have been described in detail in previous reports (1, 3, 11). It has been pointed out previously that an advantage of the metabolic clearance method is that it can be applied in nonsteady state conditions (3, 12). The total amount of albumin degraded over each period was obtained from the sum of the daily values for degradation determined by the metabolic clearance method. During the control periods, the serum albumin concentrations remained constant, and for practical purposes, it was assumed that steady state conditions, in terms of albumin metabolism, were satisfied. The quantity of albumin synthesized during these control periods was taken to be equivalent to the amount degraded. The quantity of albumin synthesized during cortisone administration was obtained from the difference between the total amount degraded and the change in the total exchangeable albumin pool.

RESULTS

Results pertaining to the animal studies are summarized in Table I. During cortisone administration a drop in serum albumin concentration occurred in seven of the eight rabbits, the mean value decreasing from 4.47 grams per cent to 3.79 grams per cent, a decrease of 11.2 per cent. Plasma volume increased in all animals with a mean value of plus 23 per cent. Mean values for albumin degradation (and albumin synthesis) during the control period determined by methods (*A*) and (*B*) were 1.51 and 1.52 grams per day, respectively. During cortisone administration the quantity of albumin degraded increased in all animals (Table I, Figure 2) to an average value of 2.4 grams per day. The total increase in albumin degradation during the period of treatment ranged from 6.8 grams to 20.0 grams, with a mean value of 13.1 grams. Since the mean total exchangeable albumin decreased only slightly from 18.1 to 17.0 grams, synthesis of albumin must have increased by an average of 12.0 grams during the experimental period. The extravascular albumin diminished during cortisone administration in all but one animal.

TABLE I

The effects of cortisone on albumin distribution and metabolism in rabbits

Rabbit	Albumin conc.		Plasma volume		Albumin degraded			Days on cortisone	Increased albumin degraded during exp. period	TEA		TEA change	Increased synthesis of albumin during exp. period	Albumin partition			
					Cont.		Exp.							Cont.		Exp.	
	Cont.*	Exp.	Cont.	Exp.	A	B	B			Cont.	Exp.			Int.	Ex.	Int.	Ex.
no.	*Gm./100 ml.*		*ml.*		*Gm./day*				*Gm.*	*Gm.*		*Gm.*	*Gm.*				
1	4.23	3.60	160	177	1.47	1.45	2.84	14	19.5	16.8	14.0	−2.8	16.8	6.7	10.1	6.4	7.6
2	4.48	4.16	147	171	1.60	1.64	2.59	16	15.4	18.5	16.0	−2.5	13.4	6.6	11.9	7.1	8.9
3	4.79	3.41	135	157	1.58	1.58	2.21	15	9.5	18.8	14.2	−4.6	4.9	6.5	12.3	5.4	8.8
4	4.37	3.80	144	178	1.40	1.42	3.09	12	20.0	17.2	18.7	+1.5	21.5	6.3	10.9	6.8	11.9
5	4.06	4.42	162	223	1.47	1.57	2.09	17	8.9	18.4	20.7	+2.3	11.2	6.6	11.8	9.8	10.9
6	4.22	4.10	184	234	1.30	1.35	2.11	17	12.9	18.3	19.0	+0.7	13.6	7.8	10.5	9.6	9.4
7	4.52	3.80	143	192	1.16	1.12	1.89	15	11.5	16.7	16.1	−0.6	10.9	6.5	10.2	7.3	8.8
8	5.05	4.45	161	191	2.04	2.03	2.39	19	6.8	20.3	17.0	−3.3	3.5	8.2	12.1	8.5	8.5
Mean	4.47	3.97	156	190	1.50	1.52	2.40	15.6	13.1	18.1	17.0	−1.1	12.0	7.1	11.0	7.8	9.2
	±0.33 (S.D.)	±0.37 (S.D.)															
Percentage	−11.2		+23				+58									+9.9	−16.4

* Abbreviations: Cont., Control period; Exp., (Experimental) during cortisone administration; A, Derived from plasma decay curve; B, Derived from metabolic clearance technique (see text); TEA, Total exchangeable albumin; Int., Intravascular albumin; Ex., Extravascular albumin.

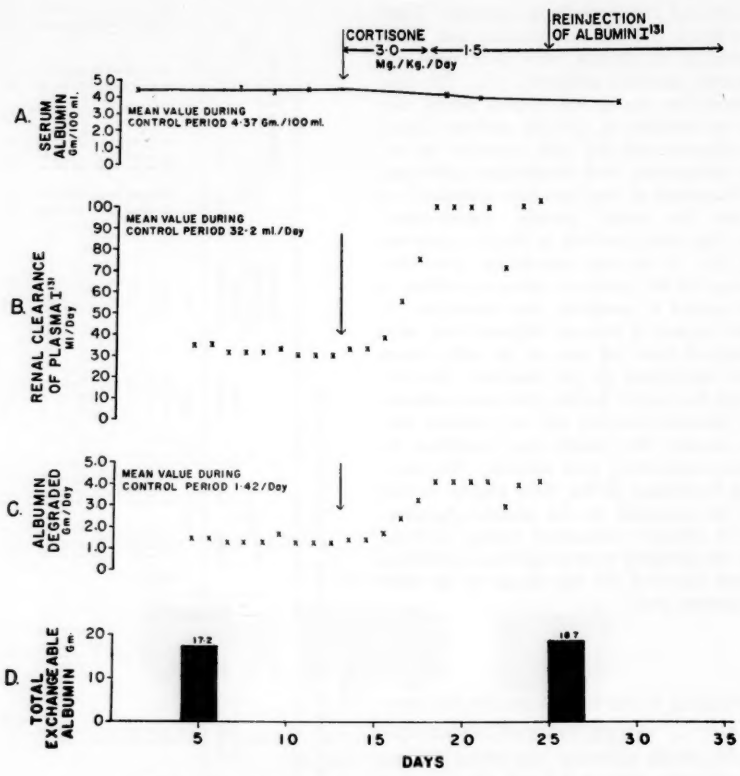

FIG. 2. TYPICAL ANIMAL EXPERIMENT, RABBIT NO. 4

Following cortisone administration, there was a slow progressive fall in serum albumin concentration (Curve A), a marked rise in the renal clearance of plasma I^{131} (Curve B) and a rise in the quantity of albumin degraded (Curve C). Total exchangeable albumin increased slightly (Bar Graphs D).

The nonfasting blood sugars ranged from 95 to 268 mg. per cent in the seven animals in which it was measured. All of the animals revealed intermittent glycosuria within 48 to 72 hours after the institution of cortisone therapy. There was no significant change in the animals' weights from the control through the experimental periods. Hematocrit values fell in all animals with a mean decrease of 11.5 per cent. Proteinuria was not present in any of the animals.

The human studies are summarized in Table II. Patient W. T. is separated from the other subjects because this patient's control value for albumin turnover (312 mg. per Kg. per day) was significantly increased above normal values. Total serum protein concentration fell in all the cases and was attributable primarily to a decrease in the globulin fraction. The changes in plasma volume were negligible except in one case (T. H.), where there was a decrease of 9 per cent. The mean rates of albumin degradation during the control period were 14.7 grams per day and 13.9 grams per day by methods (*A*) and (*B*), respectively. During the experimental period a mean value of 17.7 grams per day was obtained (method B). Albumin degradation in excess of that during the control period ranged from 32 to 88 grams with a mean value of 59 grams. Since total exchangeable albumin decreased in these four subjects by 31 grams (range, 22 to 38 grams), the calculated

TABLE II

The effects of hydrocortisone and prednisone on albumin distribution and metabolism in humans

Subject	Diagnosis	Plasma concentration				Plasma volume		Albumin degraded			Time on steroids	Increase in albumin degraded during exp. period	TEA		TEA loss	Increased synthesis of albumin during exp. period	Albumin partition			
		Total protein		Albumin				Cont.		Exp.			Cont.	Exp.			Cont.		Exp.	
		Cont.*	Exp.	Cont.	Exp.	Cont.	Exp.	A	B	B							Int.	Ex.	Int.	Ex.
		Gm./100 ml.				*ml.*		*Gm./day*			*days*	*Gm.*	*Gm.*		*Gm.*	*Gm.*				
J. O.	Osteoarthritis	7.70	6.70	5.30	5.05	2,870	2,690	15.7	14.7	19.3	19	88	378	340	38	50	156	222	135	205
J. A.	Rheumatoid arthritis	6.70	6.33	4.55	4.42	2,940	3,010	11.4	10.6	14.2	14	50	297	275	22	28	133	164	133	142
T. H.	Cerebrovascular disease, duodenal ulcer	7.82	7.32	4.82	4.82	3,400	3,070	17.6	16.7	21.9	13	68	401	357	44	24	164	237	148	209
A. V.	Osteoarthritis	7.71	6.81	4.77	4.60	3,290	3,260	14.0	13.5	15.2	19	32	365	343	22	10	157	208	151	192
Mean								14.7	13.9	17.7	16	59	360	329	31	28	152	208	143	186
W. T.	Probable collagen disease	8.19	7.89	4.25	4.28	3,160	2,940	18.0	17.7	18.0	14	3	379	376	3	0	130	249	129	247

* Abbreviations: Cont., (Control) before steroid therapy; Exp., (Experimental) during steroid therapy; A, Derived from plasma concentration curve; B, Derived from metabolic clearance technique; TEA, Total exchangeable albumin; Int., Intravascular; Ex., Extravascular.

increase in synthesis averaged 28 grams (range, 10 to 50 grams). Loss in total exchangeable albumin occurred from both intravascular and extravascular compartments, with the major fraction coming from the extravascular stores. The patients' weights remained constant, no significant change in hematocrit values was observed, and proteinuria was not observed.

Patient W. T., who showed a greater than normal albumin turnover during the control period, was ill with an acute febrile illness considered to be a "collagen disease." Following the institution of prednisone, there was a steady clinical improvement, with remission of all symptoms. However, no significant changes in albumin turnover or distribution were observed during the course of these studies.

DISCUSSION

Although an increased loss of urinary nitrogen frequently accompanies the administration of excess adrenocortical hormones (4, 6, 13–17), it has been emphasized that such excretory data only reflect the overall picture (17), for while some tissues are particularly susceptible to the wasting action of these hormones (18–23), others such as the liver, gastrointestinal tract and urogenital organs may actually show a positive nitrogen balance (13, 15, 17). The present studies indicate that serum albumin metabolism is significantly altered during the administration of certain adrenocortical hormones. There is a moderate to marked increase in the rate of albumin catabolism associated with an increase of similar magnitude in the rate of albumin synthesis, so that there results only a negligible loss of body albumin stores. These observations clearly provide no support for the thesis that these hormones exert an antianabolic effect on all protein metabolism. The small defect in albumin balance was derived mainly from the extravascular stores, a finding which has also been recorded following the administration of large doses of desiccated thyroid (3).

The alterations in albumin metabolism were not as great in the human subjects as in the rabbits. This fact probably relates both to the different drugs and dosage schedules employed. However, the findings were qualitatively the same in

both species and agree in substance with the preliminary report of Grossman, Yalow and Weston (24). Blythe and co-workers (25) have also reported, in abstract form, an increased rate of albumin degradation following the daily administration of 300 mg. of hydrocortisone to human subjects. The acutely ill patient, W. T., was probably under the influence of increased adrenocortical stimulation secondary to the active disease process, which may explain the lack of increase in this patient's already rapid albumin turnover following the administration of exogenous adrenocortical hormone.

The validity of methods employing albumin-I^{131} for the measurement of albumin metabolism and albumin distribution has been subjected to extensive critical review elsewhere (1, 2, 12). It would seem unnecessary to enter into a similar discussion here. However, it may be pointed out that McFarlane (26) has obtained almost identical values for albumin metabolism in rabbits employing albumin-I^{131} labeled *in vitro* and biosynthetically labeled albumin-C^{14}, and Berson and Yalow (12) have emphasized that available data for human subjects indicates similar rates of metabolism of albumin-I^{131} and endogenously labeled albumin-S^{35} when account is taken of reutilization of the latter label (12). A major difficulty in the use of albumin-I^{131} in the past has been in the preparation of undenatured material (1, 12, 26), but the albumin-I^{131} used in the present studies has given values almost identical to those obtained with the best preparations reported elsewhere in rabbits (27) and man (1, 2). Furthermore, since all studies in the present report employed the same dose of albumin-I^{131} for control and experimental periods, the observed changes cannot be attributed to variations in the preparations.

SUMMARY AND CONCLUSIONS

1) The distribution and metabolism of albumin employing species specific albumin-I^{131} were studied in eight female rabbits and five male patients before and during the administration of adrenocortical hormones.

2) Under the influence of cortisone, albumin degradation increased by 58 per cent in the animals and augmented albumin synthesis compensated for 91 per cent of this increased albumin destruction. In the human experiments, albumin degradation increased 27 per cent while increase in synthesis replaced 48 per cent of the albumin degraded in excess of the control values.

ACKNOWLEDGMENTS

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INTERRELATIONS BETWEEN SERUM SODIUM CONCENTRATION, SERUM OSMOLARITY AND TOTAL EXCHANGEABLE SODIUM, TOTAL EXCHANGEABLE POTASSIUM AND TOTAL BODY WATER [1]

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Although much is known about the effects of changes in electrolyte and water balance on serum sodium concentration, the quantitative relationships between body composition and the concentration of sodium in serum have not been established. Also, no definitive study has been made of the correspondence between serum sodium concentration and total serum osmolarity. It is necessary that these relationships be defined to permit classification and interpretation of abnormalities of serum sodium concentration in clinical states, as well as to extend present understanding of the effects of electrolyte equilibria and acid-base disturbances on serum sodium concentration.

It has been definitively demonstrated that no overall correlation exists between body sodium content, or external balance of sodium, and serum sodium concentration (1–4). Serum sodium concentration, of course, is not independent of loss or gain of body sodium. Excessive loss of sodium leads to a fall in serum concentration; administration of hypertonic saline elevates serum levels (5–8). Retention of water in excess of sodium has been shown to occur, and accounts, at least in part, for the hyponatremia seen after major surgery and in patients with coexistent edema and hyponatremia (3, 4, 9). This phenomenon has been demonstrated experimentally by administration of vasopressin to patients with congestive heart failure (10). An integrated account of concomitant alterations in serum sodium concentration and body composition was first achieved by Deming and Gerbode (11), who observed that changes in serum sodium concentration paralleled net changes of sodium and potassium balance in relation to water balance in patients undergoing mitral valvulotomy. The influence of potassium balance on serum sodium concentration was confirmed by correlative studies in postoperative hyponatremia and by demonstration that administration of potassium can raise the serum sodium concentration in hyponatremic patients (4, 7, 12).

The dependence of serum sodium concentration on body sodium, potassium and water content has several important implications. The magnitude and character of this dependence will modify or extend current concepts of ionic redistributions between body fluids, of osmotic gradients across cell membranes, and of the participation of hydrogen ion in the regulation of serum sodium concentration (13–17).

Simultaneous measurements of total exchangeable sodium (Na_e), total exchangeable potassium (K_e), total body water (T.B.W.), and serum electrolyte concentrations, pH and osmolarity were made in a heterogeneous group of chronically ill patients. Statistical analyses were made of the correlations of serum sodium concentration (Na_s) with serum osmolarity (π_s), Na_e/body weight, K_e/body weight, T.B.W./body weight, Na_e/T.B.W., K_e/T.B.W. and $(Na_e + K_e)$/T.B.W. These data reveal striking correlations between Na_s, π_s and $(Na_e + K_e)$/T.B.W.

METHODS

Ninety-eight patients were studied. The age, sex, state of hydration of these subjects and the clinical diagnoses are listed in Table I. Maximum heterogeneity in

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[2] During tenure as an Established Investigator of the American Heart Association.

[3] Research Fellow of the San Francisco Heart Association.

[4, 5] Research Fellows of the National Heart Institute of the United States Public Health Service.

TABLE I

Summary of clinical data

Patient	Age	Sex	Body wt.	Transudate	Diagnosis
				Heart disease	
no.	*yrs.*		*Kg.*		
1	64	M	64.1	+2	Hypertensive and arteriosclerotic cardiovascular disease
5	64	M	53.9	+2	Hypertensive and arteriosclerotic cardiovascular disease
7	67	F	85.0	+3	Arteriosclerotic heart disease
10	66	M	119.5	+4	Arteriosclerotic heart disease
11	76	M	80.0	+2	Hypertensive and arteriosclerotic heart disease
12	68	M	59.8	+1	Arteriosclerotic heart disease; bronchiectasis
18	55	M	41.8	+1	Cor pulmonale; chronic pulmonary disease
21	60	M	59.8	0	Cor pulmonale; chronic bronchitis; pneumonia
23	51	F	55.2	+2	Cor pulmonale; chronic pulmonary disease
25	67	M	47.7	+1	Cor pulmonale; chronic empyema; auricular fibrillation
26	58	M	75.8	+4	Hypertensive heart disease
27	89	M	60.9	+2	Arteriosclerotic heart disease; chronic pyelonephritis
36	72	F	38.6	0	Arteriosclerotic heart disease; cerebrovascular disease
37	79	M	62.3	+1	Hypertensive heart disease
42	71	M	44.8	0	Cor pulmonale; asthma; bronchopneumonia
47	54	M	48.6	+3	Arteriosclerotic heart disease; duodenal ulcer
49b	86	F	51.8	+1	Arteriosclerotic heart disease
51	84	M	56.4	+2	Arteriosclerotic heart disease
53	54	M	49.8	+2	Arteriosclerotic heart disease
61	71	M	58.0	+2	Arteriosclerotic heart disease
75	66	M	53.6	+2	Arteriosclerotic heart disease
78	40	M	71.4	+2	Malignant hypertension
80	82	F	63.6	+1	Arteriosclerotic heart disease
80a	75	M	86.4	+4	Arteriosclerotic heart disease
82	53	F	103.9	+1	Arteriosclerotic heart disease
90	60	M	53.2	+4	Hypertensive heart disease
93	74	M	65.9	+4	Arteriosclerotic heart disease
94	77	M	55.7	+3	Rheumatic heart disease
56	54	F	70.0	0	Essential hypertension
57	46	M	86.6	0	Essential hypertension
58	40	F	56.1	0	Essential hypertension
67a	27	F	60.9	0	Essential hypertension
94a	33	F	55.0	0	Essential hypertension
				Liver disease	
2	50	M	67.3	+4	Cirrhosis
3	47	M	80.0	+2	Cirrhosis
6	51	M	41.8	+3	Cirrhosis
8	51	M	37.7	+2	Cirrhosis
13	66	M	88.4	0	Cirrhosis
16	74	M	101.3	+4	Cirrhosis
19	28	F	39.6	+1	Cirrhosis
20	45	M	50.9	+2	Cirrhosis
22	37	F	39.8	+1	Cirrhosis
24	59	M	67.0	+3	Cirrhosis
29	66	F	82.5	0	Cirrhosis
30	51	M	78.8	+3	Cirrhosis
35	43	F	51.8	+3	Cirrhosis
38	67	M	79.3	+4	Cirrhosis
43	56	M	112.5	0	Cirrhosis
44b	43	F	56.8	+3	Cirrhosis
48a	67	M		+4	Cirrhosis
50	39	F	65.7	+3	Cirrhosis
60	27	F	55.7	+1	Cirrhosis
66	48	M	55.5	+2	Cirrhosis
67	85	M		0	Cirrhosis
71	50	F		+3	Cirrhosis
73	48	F	57.7	+4	Cirrhosis
79	64	M	78.2	+4	Cirrhosis
81	64	M	81.4	+4	Cirrhosis
84	39	M	84.6	+4	Cirrhosis
86	51	M	66.4	+3	Cirrhosis
87	46	M	76.8	+2	Cirrhosis
88	50	M	60.0	+3	Cirrhosis
89	59	M	67.7	+4	Cirrhosis
91	42	M	64.0	+2	Cirrhosis
92	45	M	60.0	+4	Cirrhosis

TABLE I—*Continued*

Patient	Age	Sex	Body wt.	Transudate	Diagnosis
				Kidney disease	
no.	*yrs.*		*Kg.*		
17	47	M	70.7	+3	Nephrotic syndrome
28	58	M	47.7	0	Chronic glomerulonephritis; gastric ulcer
33	58	M	51.1	+3	Chronic glomerulonephritis
62	36	M	69.8	+4	Diabetic glomerulosclerosis
74	40	F	40.0	+2	Chronic pyelonephritis
				Lung disease	
31	69	M	48.0	0	Pulmonary emphysema
48b	48	M	52.3	0	Pulmonary tuberculosis; cirrhosis of liver
63	29	F	51.3	+1	Pulmonary tuberculosis
64	62	M	52.1	+3	Pulmonary tuberculosis; cirrhosis of liver; diabetes
59	64	M	60.2	+3	Carcinoma of lung, postoperative
				Gastrointestinal disease	
4	68	M	86.9	0	Gastric ulcer
9	45	M	69.2	0	Duodenal ulcer
44a	60	M	39.8	+2	Rectal carcinoma, postoperative
46	78	M	44.9	+1	Duodenal ulcer; chronic pyelonephritis
				Neurological disease	
40	78	F	41.4	0	Cerebrovascular disease
41	73	M	60.5	0	Cerebrovascular disease
65	45	M		+2	Transection of spinal cord
68	70	M		0	Subdural hematoma
72	77	F	43.2	0	Subdural hematoma, postoperative
77	55	F	39.1	0	Cerebrovascular disease
83	72	F	38.6	0	Cerebrovascular disease
34	90	M	43.6	0	Cerebral arteriosclerosis
				Miscellaneous	
14	67	F	59.5	0	Multiple abscesses
15	72	M	59.3	0	Cellulitis
32	39	M	168.2	+2	Obesity
39	28	F	36.4	0	Adrenal insufficiency
45	74	M	57.0	0	Senility; anemia
52	70	M	50.9	0	Chronic bromide intoxication
55	41	M	53.6	0	Primary aldosteronism
70	62	M	61.8	0	Panhypopituitarism
76	62	M	62.5	0	Panhypopituitarism
54	49	M	63.9	+4	Carcinomatosis, primary unknown
69	61	F	47.7	+4	Carcinoma of cervix with peritoneal metastases

clinical and metabolic status was sought to assure that any correlations between body composition and serum electrolyte concentrations would have general validity. The group consisted of 33 patients with a variety of heart diseases, 32 patients with cirrhosis of the liver, 5 patients with renal disease, 5 patients with pulmonary disease, 4 patients with gastrointestinal disease, 8 patients with neurological disease and 11 patients with other illnesses, including panhypopituitarism, adrenal insufficiency and primary aldosteronism. Sixty-six of these patients had one to four plus edema by clinical criteria.

All subjects were given an analyzed diet which contained 10 mEq. of sodium and 28 mEq. of potassium each day for one or two days prior to and for the three days of study. Food and fluid were withheld for the six hour period of D_2O equilibration.

The sequence and technique of isotope administration and collection of urine and blood for isotope assay have been described previously (18, 19). No subject received more than 360 microcuries of K^{42} and 150 microcuries of Na^{24}. The periods of isotope equilibration were 40 hours for K^{42}, 24 hours for Na^{24} and 6 hours for D_2O. These periods were previously shown to be adequate for isotope equilibration, even in very edematous subjects (19). Blood was drawn from the femoral artery just before injection of Na^{24}. Glycolysis in these samples was inhibited either by the addition of 0.5 ml. of saturated NaF or by icing.

The analytical methods for isotope assay have been described in earlier papers (18, 19). Serum osmolarity (π_s) was estimated by freezing point depression with a thermistor probe, using NaCl standards (20).[6] Sodium

[6] Freezing point depression actually is a measure of osmolality. Since osmolality is virtually equivalent to osmolarity at constant temperature, however, the latter term has been used in this communication to provide units comparable with the electrolyte concentration data.

TABLE II

Summary of laboratory data: Serum values

Patient	Na'_s	K'_s	Cl_s	$(HCO_3)_p$	Arterial pH	NPN*	Glucose	π_s	π'_s
				Heart disease					
no.	*mEq./L.* of serum water	*mEq./L.* of serum water	*mEq./L.*	*mEq./L.*		*mg. %*	*mg. %*	*mOsm./L.*	*mOsm./L.*
1	155.6	4.58	104.6	27.5	7.47	24	89	289.2	275.7
5	142.7	4.64	96.3	31.4	7.40	29	93	274.6	259.0
7	153.1	4.03	103.2	32.4	7.41	17	118	287.2	274.5
10	149.0	4.77	99.3	23.9	7.47	17	112	276.9	264.6
11	147.7	4.19	104.0	25.2	7.46	24	91	285.2	271.5
12	130.1	4.91	86.2	27.8	7.51	18	97	245.3	233.5
18	147.9	4.72	98.7	30.9	7.35	17	101	280.9	269.2
21	136.9	4.89	84.9	40.4	7.34	21	86	271.1	258.8
23	137.3	5.44	85.2	35.7	7.25	43	100	274.0	253.1
25	144.5	4.19	94.7	36.4	7.34	12	87	275.7	266.6
26	137.9	6.49	102.9	19.0	7.37	72	116	288.6	256.5
27	120.6	5.17	86.8	12.2	7.19	126	149	273.4	214.6
36	145.5	2.52	93.1	34.0	7.45	16	114	272.4	260.4
37	146.1	4.68	109.7	21.8	7.49	45	100	292.2	270.6
42	132.7	4.38	95.8	25.9	7.34	29	111	264.7	248.1
47	138.5	4.55		12.8	7.48	142	127	311.0	256.4
49b	141.6	2.85		25.3	7.54	21	198	272.0	253.5
51	120.3	4.77		21.5	7.58	11	80	232.0	224.0
53	117.6	4.39	72.6	26.8	7.49	98	95	248.0	208.0
61	123.4	4.99	77.2	29.2	7.48	10	100	228.0	218.8
75	126.1	5.92	88.5	22.8	7.52	22	144	254.5	238.5
78	140.8	3.36		25.9	7.39	76	83	290.0	258.2
80	123.0	4.91	83.5	22.4	7.49	23	120	243.4	228.5
80a	142.3	4.35				32	76	281.0	265.0
82	159.0	4.06				13	116	298.0	287.0
90	139.4	4.28		28.0	7.45	48	142	289.0	264.0
93	145.0	3.39		27.5	7.44	36	97	283.0	264.7
94	148.7	5.16		28.4	7.27	83	112	306.0	270.2
56	150.8	4.60				49	100	283.8	260.7
57	148.0	4.97				27	98	286.1	271.1
58	143.2	3.97				20	108	277.0	263.9
67a	152.6	4.58				15	108	283.0	271.6
94a	150.4	3.88				15	89	275.0	265.0
				Liver disease					
2	146.0	3.09	108.2	17.1	7.48	34	121	280.0	261.2
3	145.7	3.94	106.5	21.7	7.52	17	101	275.0	263.3
6	128.5	5.21	84.4	28.5	7.38	17	117	245.8	233.2
8	143.5		98.1	23.9	7.46	22	85	276.1	263.5
13	150.0	4.75	95.3	28.2	7.38	95	148	303.6	261.5
16	140.8	3.21	101.8	22.7	7.45	44	118	280.3	258.0
19	139.5	3.70	95.0	26.3	7.39	11	66	262.3	254.7
20	127.9	4.90	94.1	19.1	7.39	31	116	258.4	240.9
22	141.3	4.05	100.8	23.9	7.40	13	83	269.2	260.0
24	133.9	3.91	98.6	21.5	7.45	45	75	265.0	244.7
29	141.8	3.11	101.1	25.9	7.40	19	112	274.5	261.5
30	147.2	3.58	108.3	24.9	7.47	19	86	279.8	268.2
35	139.1	4.44	105.6	18.8	7.48	30	104	274.0	257.5
38	138.1	3.97	101.7	22.7	7.47	16	109	267.4	255.6
43	141.2	4.03				23	112	277.1	262.7
44b	131.6	3.93	94.3	26.5	7.49	12	70	252.1	243.9
48a	108.9	2.54				58	80	227.6	202.5
50	139.6	3.26		23.0	7.54	23	100	257.0	243.2
60	156.4	6.49	125.6	8.1	7.35	145	317	360.0	290.6
66	151.2	4.36	104.0	23.5	7.47	14	86	285.0	275.0
67	170.6					102	164	356.0	310.0
71	112.0	4.82	76.6	20.7	7.28	72	160	243.0	208.4
73	130.7	3.72	85.6	32.9	7.44	32	502	285.0	245.7
79	124.0	3.56				19	127	240.5	226.6
81	144.4	3.96				19	90	280.0	268.0
84	144.4	4.13				22	122	276.5	261.8
86	144.6	6.15				103	95	304.0	262.0
87	126.5	3.54		21.1	7.36	173	150	295.0	225.0
88	141.2	3.80		24.7	7.54	20	125	268.5	254.5
89	136.4	3.96		25.6	7.54	19	120	262.0	248.5
91	144.3	4.30				18	92	271.0	259.5
92	135.7	4.41				16	94	266.0	255.1

* Nonprotein nitrogen.

TABLE II—*Continued*

Patient	Na'_s	K'_s	Cl_s	$(HCO_3)_p$	Arterial pH	NPN*	Glucose	π_s	π'_s
				Kidney disease					
no.	*mEq./L. of serum water*	*mEq./L. of serum water*	*mEq./L.*	*mEq./L.*		*mg. %*	*mg. %*	*mOsm./L.*	*mOsm./L.*
17	146.4	7.00	103.6	15.3	7.28	100	87	304.8	264.3
28	144.0	3.90	103.3	19.3	7.31	139	109	317.3	261.6
33	111.2	4.76		22.4	7.40	127	136	260.0	207.0
62	134.3	5.13	76.3	28.1	7.49	165	157	309.0	241.4
74	137.3	4.54		17.7	7.30	212	139	310.0	226.6
				Lung disease					
31	146.3	5.25	90.6	31.5	7.41	37	109	280.3	261.0
48b	134.6	3.67		27.1	7.52	13	100	241.0	230.8
63	145.7	4.37	96.5	26.4	7.45	9	121	266.0	256.0
64	125.5	3.80	76.6	32.0	7.54	16	295	250.0	228.0
59	134.1	5.55	86.1	28.6	7.59	42	137	268.0	245.0
				Gastrointestinal disease					
4	147.3	4.44	100.7	29.0	7.46	23	103	277.6	263.7
9	148.7	3.97	106.2	24.0	7.46	33	149	282.8	262.7
44a	116.2	6.32	89.7	11.5	7.31	39	150	239.0	216.8
46	155.0	4.69		15.7		128	127	320.0	267.2
				Neurological disease					
40	143.9	3.65	113.7	25.9	7.44	30	91	278.0	262.2
41	133.1	4.48	116.8	23.4	7.42	13	127	256.7	245.0
65	131.3	4.78	80.0	24.5	7.55	30	139	254.0	235.0
68	192.5					238	367	450.0	345.0
72	135.8	4.09	92.0	30.4	7.50	15	95	258.0	247.3
77	158.1	2.28	103.8	34.1	7.47	34	100	301.7	284.0
83	161.5	3.97				34	102	317.0	304.2
34	144.5	4.40	101.5	26.8	7.39	36	136	286.5	266.0
				Miscellaneous					
14	147.3	4.35	103.7	28.1	7.41	16	83	278.7	268.4
15	146.1	4.62	106.1	25.8	7.41	27	100	280.9	265.7
32	148.3	4.02	97.1	34.3	7.35			283.7	
39	138.5	4.13	111.4	22.8	7.44	20	95	262.0	249.6
45	129.5	5.14	94.9	22.4	7.43	24	98	252.9	238.9
52	142.3	3.80		33.2	7.55	25	105	263.0	248.0
55	154.6	3.50	98.9	31.7	7.45	30	90	292.0	276.0
70	119.7	4.48	87.6	17.5	7.58	9	78	224.0	216.5
76	135.0	4.65				23	83	262.0	249.2
54	132.3	5.63		18.7	7.43	61	103	273.0	245.0
69	123.3	6.87	87.3	22.0	7.52	14	97	236.0	225.6
Mean	140.1	4.40	96.4	25.0	7.44	45.3	120	276.6	254.5
Range	108.9–192.5	2.28–7.00	72.6–125.6	8.1–40.4	7.19–7.59	9–238	66–502	224.0–450.0	202.5–345.0

and potassium were measured in dilutions of urine and serum with a lithium internal-standard flame photometer. Serum water was determined gravimetrically by drying 1 ml. aliquots, delivered from calibrated pipettes, at 104° C. for 72 hours. Serum chloride (Cl_s) was estimated by electrometric titration (21), plasma glucose by colorimetry (22) and plasma nonprotein nitrogen (NPN) by a modification of the Folin-Wu method (23). Total CO_2 content of arterial plasma was determined by the manometric method of Van Slyke (24). Arterial blood pH was measured at 37° C. with a Beckman model G pH meter, calibrated with buffer standards prepared from high purity salts provided by the National Bureau of Standards. All chemical determinations were done in duplicate or triplicate.

Calculations, statistics, and analytical error. Standard formulas were used in calculating specific activities, Na_e, K_e and T.B.W., including the appropriate corrections for isotope excretion in the urine (25–27). The measured Na_e was back corrected to the time of determinations of K_e and T.B.W. by metabolic balance for sodium during the period of Na^{24} equilibration. This correction was applied to provide simultaneous estimates of Na_e, K_e and T.B.W. since Na_e was measured 24 hours after measurement of K_e and T.B.W.

The osmotic contributions of glucose and NPN were

TABLE III

Summary of laboratory data: Body composition

Patient	Na_e	Na_e/Body wt.	K_e	K_e/Body wt.	T.B.W.	T.B.W./Body wt.	$\frac{Na_e + K_e}{T.B.W.}$
			Heart disease				
no.	*mEq.*	*mEq./Kg.*	*mEq.*	*mEq./Kg.*	*L.*	*%*	*mEq./L.*
1	3,122	48.7	2,454	38.3	34.1	53.1	163.8
5	3,189	59.2	1,963	36.5	33.8	62.7	152.5
7	4,876	57.4	1,936	22.8	44.8	52.7	152.2
10	6,983	58.4	3,293	27.6	69.6	58.3	147.6
11	3,812	47.7	2,681	33.5	39.9	50.3	162.9
12	2,359	39.5	2,599	43.5	35.0	58.5	141.7
18	1,953	46.7	1,862	44.5	24.9	59.5	153.3
21	3,149	52.7	2,168	36.3	33.5	56.1	158.5
23	3,486	63.2	1,704	30.9	36.3	65.7	143.1
25	3,051	64.2	1,665	34.9	30.9	64.8	154.6
26	5,116	67.5	2,772	36.6	49.5	65.9	159.3
27	2,880	47.3	1,639	26.9			
36	2,230	57.8	1,279	33.1	23.6	61.2	148.5
37	2,971	47.7	1,986	31.9	32.4	52.0	150.6
42	2,495	55.7	1,650	36.9	29.8	66.5	139.3
47	3,046	62.7	1,750	36.0	31.6	65.0	151.9
49b	3,176	61.3	1,245	24.0	30.3	58.4	146.1
51	3,385	60.0	1,424	25.2	36.8	65.2	130.8
53	3,734	75.0	1,211	24.3	37.6	75.4	131.6
61	3,451	59.5	2,048	35.3	40.0	68.9	137.5
75	3,659	68.2	1,699	31.7	38.9	72.5	137.8
78	3,702	51.9	3,723	52.1	50.4	70.5	147.5
80	1,957	30.8	1,164	18.3	24.0	37.7	130.1
80a	6,400	74.1	2,213	25.6	60.6	70.1	142.3
82	3,105	29.9	3,021	29.0	39.2	37.7	156.5
90	3,733	70.2	1,571	29.5	36.3	68.3	146.0
93	4,770	72.4	1,937	29.3	47.2	71.6	142.1
94			1,804	32.4	39.2	70.4	
56	2,227	31.8	2,227	31.8	28.5	40.8	156.1
57	3,447	39.8	4,222	48.9	45.6	52.7	168.1
58	2,180	38.7	2,063	36.8	27.9	49.7	152.0
67a	2,363	38.8	2,281	37.5	29.2	48.0	158.2
94a	2,276	41.4	1,882	34.2	27.9	50.8	148.8
			Liver disease				
2	4,152	61.7	1,785	26.5	39.3	58.3	151.2
3	3,541	45.1	3,205	40.1	44.5	55.6	151.7
6	2,417	57.9	1,438	34.4	26.9	64.3	143.5
8	2,906	77.1	1,044	27.7			
13	3,327	37.6	2,889	32.7			
16	5,153	50.9	2,350	23.2	50.1	49.5	149.6
19	2,228	56.3	1,203	30.4	22.7	57.4	151.2
20	2,359	46.3	1,665	32.7	27.7	54.2	145.5
22	1,995	50.2	1,266	31.8	22.0	55.3	148.2
24	3,511	52.4	2,140	31.9	37.4	55.4	151.0
29	2,716	32.9	2,265	27.5	32.5	39.4	153.2
30	5,386	68.4	2,819	35.8	52.3	66.3	156.9
35	3,772	72.8	1,525	29.4	35.5	68.6	149.1
38	4,236	79.3	2,624	33.1	45.0	56.8	152.3
43	3,337	29.7	3,637	32.3	45.4	40.4	153.6
44b	2,941	51.8	1,683	29.6	32.6	57.3	142.1
48a							
50	3,376	51.4	2,133	32.5	36.0	54.8	153.0
60			1,665	29.9	23.7	42.6	
66	3,085	55.6	1,816	32.7	32.6	58.7	150.5
67							
71			1,504		38.1		
73			1,802	31.2	32.7	56.7	
79	4,300	55.0	1,680	21.5	43.5	55.7	137.3
81	5,510	67.7	2,308	28.4	52.1	64.0	150.1
84	5,470	64.7	2,775	32.8	55.6	65.7	148.4
86	2,943	44.4	2,487	37.5			
87	2,852	37.1	2,521	32.8	40.0	52.1	134.3
88	3,284	54.7	2,197	36.6	37.9	63.2	144.5
89	3,116	46.0	1,762	26.0	35.6	52.6	137.2
91	3,308	51.7	2,415	37.7	38.3	59.9	149.3
92	3,103	51.7	2,349	39.2	38.0	63.4	143.4

TABLE III—*Continued*

Patient	Na_e	Na_e/Body wt.	K_e	K_e/Body wt.	T.B.W.	T.B.W./Body wt.	$\frac{Na_e + K_e}{T.B.W.}$
			Kidney disease				
no.	*mEq.*	*mEq./Kg.*	*mEq.*	*mEq./Kg.*	*L.*	%	*mEq./L.*
17	4,899	70.8	2,510	35.5	47.6	67.3	155.7
28	2,874	60.2	2,017	42.3	31.5	65.9	155.4
33	2,839	55.5	1,671	32.7	36.0	70.4	125.3
62	5,062	72.6	2,884	41.3	56.4	80.8	140.8
74	2,247	56.2	1,595	39.9	25.4	63.5	151.2
			Lung disease				
31	2,257	47.1	2,105	43.9	27.8	58.0	156.7
48b	2,411	46.1	1,771	33.9	29.3	56.0	142.7
63	2,479	48.3	2,035	39.7	29.8	58.0	151.6
64	3,602	69.2	1,569	30.1	37.0	71.2	139.8
59			2,196	36.5	38.7	64.6	
			Gastrointestinal disease				
4	3,511	40.6	3,427	39.4	43.8	50.4	158.4
9	3,040	43.9	3,126	45.2	37.8	54.6	163.1
44a			1,313	33.0	25.2	63.2	
46	3,451	76.8	1,608	35.8	31.3	69.6	160.8
			Neurological disease				
40	2,146	51.9	1,218	29.5	22.2	53.7	151.5
41	2,446	40.5	1,805	29.9	29.9	49.4	142.4
65	2,752		2,404		34.8		148.3
68							
72	2,110	48.8	1,667	38.6	26.0	60.1	145.6
77	2,867	73.3	1,097	28.1	25.5	65.2	155.4
83	3,103	80.3	1,291	33.3	25.1	65.0	175.1
34	2,287	52.5	1,491	34.2	25.2	57.8	149.9
			Miscellaneous				
14	2,624	44.1	2,040	34.3	30.2	50.7	154.7
15	2,646	44.6	2,112	35.6	32.3	55.1	147.1
32	5,247	31.2	5,444	32.4	69.8	41.5	153.3
39	1,228	33.8	1,358	37.4	18.0	49.4	143.9
45	2,707	47.5	1,996	35.0	32.1	56.4	146.4
52	3,294	64.7	2,180	42.8	35.2	69.1	155.6
55	2,710	50.6	2,504	46.7	33.8	63.0	154.4
70	2,479	40.1	2,039	33.0	35.6	57.6	126.9
76	2,645	42.3	2,180	34.9	34.4	55.0	140.3
54	3,782	59.2	3,006	47.0	46.1	72.1	147.4
69	2,037	43.4	1,611	33.7	27.0	56.6	135.2
Mean	3,263	53.2	2,099	33.9	36.1	58.9	148.6
Range	1,228–6,983	29.7–80.3	1,044–5,444	18.3–52.1	18.0–69.8	37.7–80.8	125.3–175.1

evaluated by measuring the freezing point depression of standard solutions of glucose and urea. The observed depression of freezing point was directly proportional to the molar concentrations up to 500 mg. per 100 ml. of glucose and 300 mg. per 100 ml. of urea. The "corrected" serum osmolarity (π'_s) in mOsm. per liter was therefore calculated as:

$$\pi'_s = \pi_s - \frac{G_p}{18} - \frac{NPN_p}{2.8}, \qquad 1)$$

where π_s is the measured serum osmolarity, G_p is the plasma glucose concentration in mg. per 100 ml. and NPN_p is the plasma NPN concentration in mg. per 100 ml. The factor 1/2.8 is derived from the mean nitrogen content of NPN (28) and is based on the assumptions that, like urea, the other components of NPN have an activity coefficient of 1.0 at physiological concentrations and that the relative amounts of each constituent of NPN are constant, regardless of absolute values. The "corrected" serum sodium concentration (Na'_s), expressed as mEq. per liter of serum water, is calculated from the measured serum sodium concentration and serum water content, since spuriously low serum sodium concentrations may be recorded when the lipid or lipoprotein content of serum is large (29).

Conventional statistical equations were used to calculate standard deviation (σ), correlation coefficient (r) and sample standard deviation from regression ($S_{y.x}$) (30). The regression equations were obtained by the method of

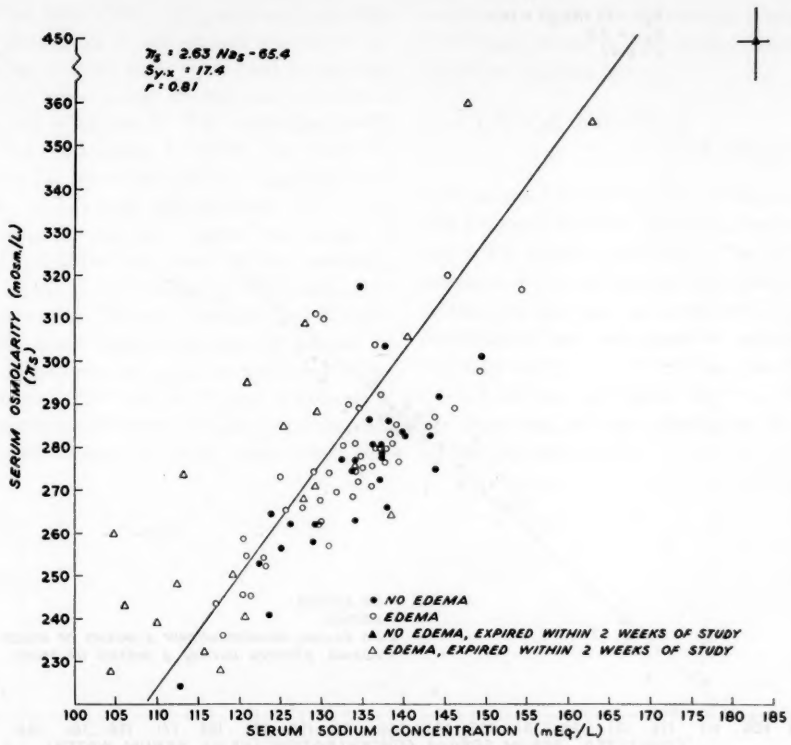
π_s = 2.63 Na_s - 65.4
$S_{y.x}$ = 17.4
r = 0.81
SERUM OSMOLARITY (mOsm./L.) (π_s)
SERUM SODIUM CONCENTRATION (mEq./L.)
• NO EDEMA
○ EDEMA
▲ NO EDEMA, EXPIRED WITHIN 2 WEEKS OF STUDY
△ EDEMA, EXPIRED WITHIN 2 WEEKS OF STUDY

FIG. 1. THE RELATION BETWEEN SERUM OSMOLARITY AND SERUM SODIUM CONCENTRATION

The statistical data were calculated as described in the text. The regression is assumed to be linear.

least squares, and the probability (p) of a correlation coefficient being obtained by chance was evaluated by the t test (30). Some of the correlations encountered were very high. The possibility was therefore considered that these very high correlations might represent perfect relationships masked only by errors of measurement. This possibility was explored in the highly significant regression relations by correcting the correlation coefficients for attenuation (31). The correlation coefficient corrected for attenuation is an estimate of the correlation between two variables free of errors of measurement. The formula for this correction is:

$$r'_{y.x} = \frac{r}{\sqrt{r_{y.y} \cdot r_{x.x}}}, \qquad 2)$$

where $r'_{y.x}$ is the amended correlation coefficient, r is the correlation coefficient computed from the data and:

$$r_{y.y} = 1 - \frac{\sigma^2_{y.m}}{\sigma^2_y} \qquad 3)$$

$$r_{x.x} = 1 - \frac{\sigma^2_{x.m}}{\sigma^2_x} \qquad 4)$$

$_{y.m}$ is the standard deviation of reproducibility of measurement of the y parameter and $_{x.m}$ is the standard deviation of reproducibility of measurement of the x parameter. $_y$ and $_x$ are the standard deviations of the observed values. To compute $_{x.m}$ for the parameter of the form (a + b)/c, the following formula was used:

$$\sigma^2_{x.m} = \left[\frac{\sigma^2_{a.m} + \sigma^2_{b.m} + \bar{x}^2 \cdot \sigma^2_{c.m}}{\bar{c}^2}\right], \qquad 5)$$

where x = (a + b)/c. This formula assumes that the errors of determination in a, b, and c are unrelated to one another, which is a reasonable assumption.

Errors of measurement were evaluated from n number of observations on pooled specimens or by serial estimations in given subjects. The standard deviations of reproducibility were as follows: $\sigma_{Na's.m}$ = 1.05 mEq. per liter (n = 21); $\sigma_{\pi's.m}$ = 1.44 mOsm. per liter (n = 16); $\sigma_{Na_s.m}$ = 65 mEq. (n = 20); $\sigma_{K_s.m}$ = 57 mEq. (n = 23); $\sigma_{T.B.W.m}$ = 0.71 liter (n = 28). $\sigma_{Na_s.m}$, $\sigma_{K_s.m}$ and $\sigma_{T.B.W.m}$ were calculated from previously established values using the standard formula $\sigma^2_m = \frac{\Sigma d^2}{2n}$ (19). These estimates are slightly lower than previously published values (32, 33).

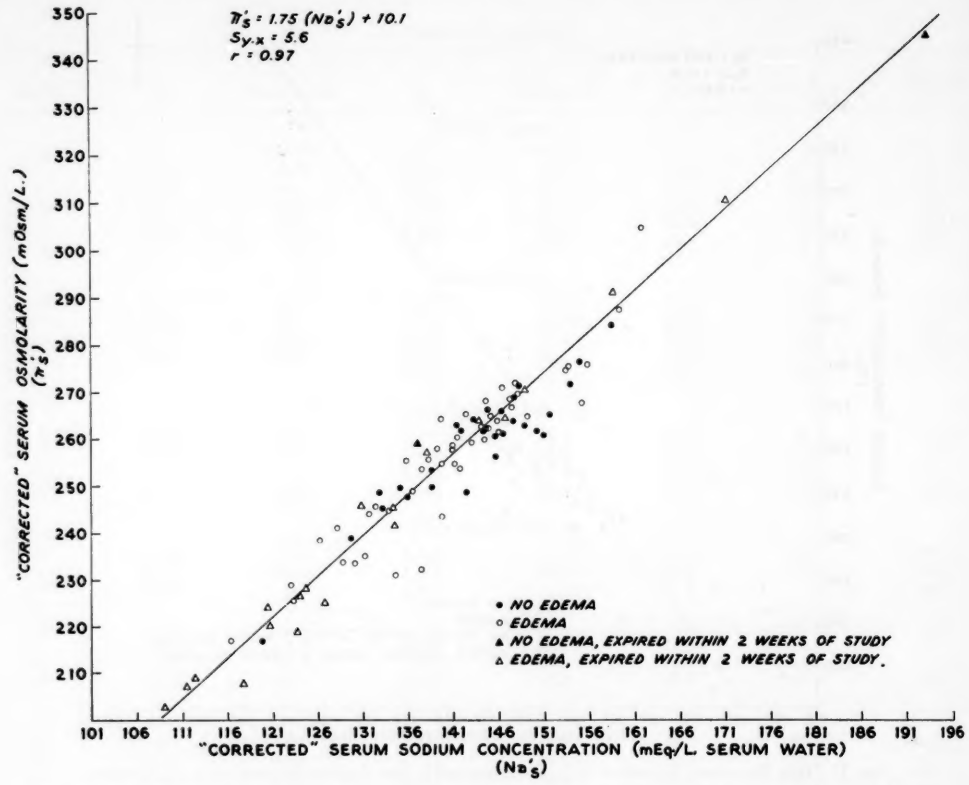

FIG. 2. THE RELATION BETWEEN "CORRECTED" SERUM OSMOLARITY AND THE "CORRECTED" SERUM SODIUM CONCENTRATION

The regression is assumed to be linear. See text for calculations of "corrected" serum osmolarity and "corrected" serum sodium concentration.

RESULTS

The data obtained in this study are listed in Tables II and III. The heterogeneity of the subjects is indicated by the wide range of values: Na'_s varied from 108.9 to 192.5 mEq. per liter of serum water, K'_s from 2.28 to 7.00 mEq. per liter of serum water, Cl_s from 72.6 to 125.6 mEq. per liter, $(HCO_3)_p$ from 8.1 to 40.4 mEq. per liter, arterial blood pH from 7.19 to 7.59 units, NPN from 9 to 238 mg. per 100 ml. of plasma, and glucose from 66 to 502 mg. per 100 ml. of plasma.

Serum osmolarity and serum sodium concentration

The total osmotic pressure of any fluid is the sum of the partial pressures of solute and may be expressed as equivalent osmotic concentrations or milliosmols. Sodium, the principal extracellular cation, must influence serum osmolarity considerably. Its quantitative contribution is depicted in Figure 1. While the correlation coefficient of the regression of total serum osmolarity with respect to serum sodium is highly significant ($r = 0.81$, $p < 0.001$), the large sample standard deviation from regression ($S_{y.x} = 17.4$ mOsm. per liter) indicates that other substances probably contribute significantly to π_s. The value of the amended correlation coefficient r′ (0.82) indicates that errors of measurement contributed little to the observed scatter. These data corroborate the findings reported by Talso, Spafford, Ferenzi and Jackson in patients with congestive heart failure and cir-

rhosis of the liver (34). The estimated contributions of plasma NPN and plasma glucose to serum osmotic activity were subtracted to evaluate the separate relationship of Na'_s to π'_s; this is demonstrated in Figure 2. The correlation coefficient of this relationship, $r = 0.97$ (see Table IV and Figure 2), shows the primary dependence of π'_s on Na'_s, as has long been assumed (35). The data in patients who died within two weeks of study did not differ from those in the remaining patients. This is in contrast to the findings of Rubin, Braveman, Dexter, Vanamee and Roberts (36), who noted hyperosmolarity of plasma in critically ill patients in excess of measured solute concentration. The data in Figure 2 indicate a linear dependence of serum osmolarity on the concentration of sodium in serum water when corrections are made for the osmotic contributions of NPN and glucose. Total serum osmolarity (π_s), therefore, is given by:

$$\pi_s = 1.75\ Na'_s + 0.0556\ G_p + 0.357\ NPN_p + 10.1 \quad 6)$$

with an amended correlation coefficient, $r' = 0.98$, and a sample standard deviation from regression, $S_{y.x} = 5.6$ mOsm. per liter. The values of the intercept of 10.1 mOsm. per liter and of $S_{y.x}$ of 5.6 mOsm. per liter can be attributed to the osmotic contributions and variations in concentrations of the salts of K^+ (~ 7.0 mOsm. per liter), Ca^{++} (~ 1.5 mOsm. per liter), Mg^{++} (~ 0.5 mOsm. per liter) and of trace substances (~ 1.0 to 3.0 mOsm. per liter) (37).

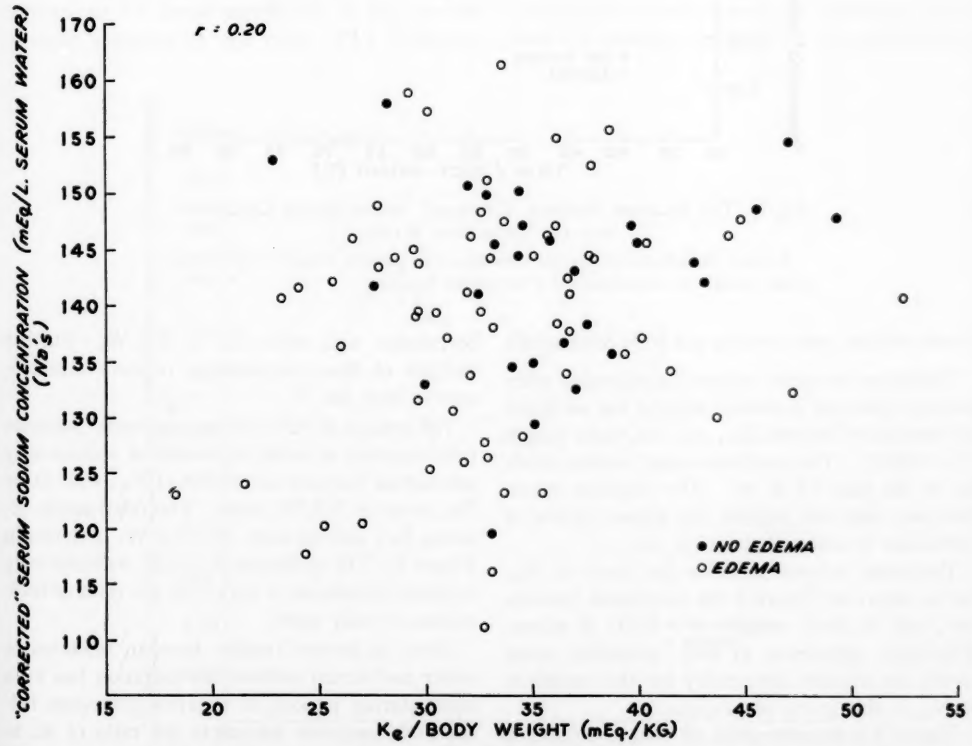

FIG. 3. THE RELATION BETWEEN THE "CORRECTED" SERUM SODIUM CONCENTRATION AND TOTAL EXCHANGEABLE POTASSIUM

The correlation is equally minor for both edematous and nonedematous subjects and does not justify calculation of a regression equation.

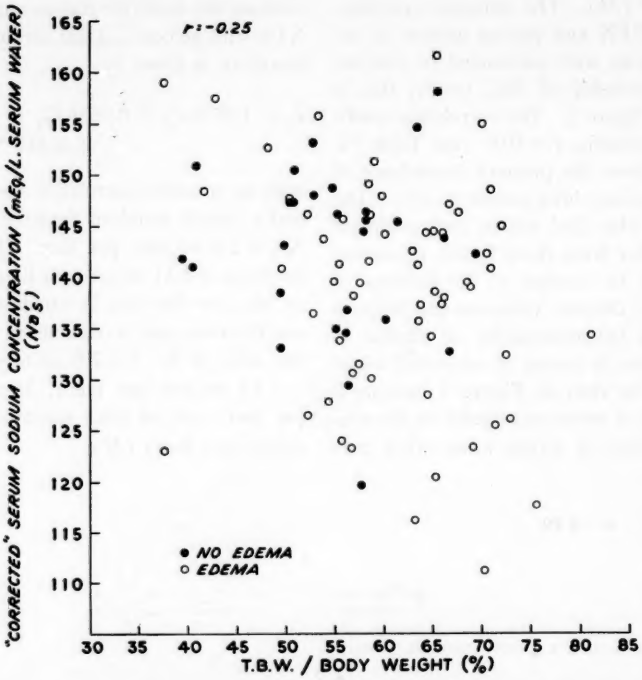

FIG. 4. THE RELATION BETWEEN "CORRECTED" SERUM SODIUM CONCENTRATION AND TOTAL BODY WATER

Neither the edematous nor the nonedematous patients shows a correlation great enough for calculation of a regression equation.

Serum sodium concentration and body composition

Variations in serum sodium concentration often parallel variations in sodium balance, but we found no correlation between Na'_s and Na_e/body weight ($r = 0.003$). This confirms many similar studies in the past (1–4, 6). The negative result, however, does not explain the known effects of alterations in sodium balance on Na_s.

Potassium balance modifies the level of Na_s, but as shown in Figure 3 the correlation between Na'_s and K_e/body weight ($r = 0.20$) is minor. Therefore, differences in body potassium alone would not account appreciably for the variations in Na_s in this group of subjects.

Figure 4 is a scattergraph of Na'_s as a function of total body water expressed as per cent of body weight. The coefficient, $r = -0.25$, indicates a minor negative correlation. Variations in body fat in these subjects may account in part for the poor correlation with either K_e or T.B.W. Further analysis of these relationships requires measurement of body fat.

The concept of "dilution hyponatremia" suggests that retention of water in excess of sodium may account for the poor correlation of Na'_s with either Na_e alone or T.B.W. alone. The relationship between Na'_s and the ratio Na_e/T.B.W. is shown in Figure 5. The coefficient, $r = 0.27$, indicates only minimal correlation of Na'_s with the ratio of body sodium to body water.

Since an inverse relation between retention of water and serum sodium concentration has been noted during periods of negative potassium balance, Na'_s may be related to the ratio of K_e to T.B.W. Figure 6 is a plot of Na'_s and the ratio K_e/T.B.W., which shows a modest correlation ($r = 0.40$). The choice of coordinates probably does not conceal any closer correlations since log-

log plots of Na'_s to Na_e/T.B.W. and to K_e/T.B.W. are equally disappointing.

The partial correlation of Na'_s with Na_e/T.B.W. and with K_e/T.B.W., as well as the evidence that body sodium, body potassium and body water affect Na_s, suggests that osmotic adjustments between body fluids may determine the relationship of Na_s to body composition (7, 11, 38–41). The precise correlation between Na'_s and π'_s may be paralleled by a similar correlation between intracellular osmolarity and intracellular K^+ concentration. The ratio $(Na_e + K_e)$/T.B.W., therefore, may be a primary determinant of Na'_s, provided that water is passively distributed across cell membranes. This hypothesis is tested in Figure 7. The high degree of correlation between Na'_s and $(Na_e + K_e)$/T.B.W. is obvious and in striking contrast to the lesser correlation of Na'_s to the separate elements in this ratio. The coefficient $r = 0.83$ is especially impressive since there must be a propagation of methodological errors in calculating this ratio. Figure 8 demonstrates the same high degree of correlation between π'_s and $(Na_e + K_e)$/T.B.W., and Table IV lists the statistical data describing these relations. The likelihood that these findings are chance phenomena is extremely small. Indeed, the correlation coefficents [$r = 0.83$ and $r' = 0.92$ for Na'_s versus $(Na_e + K_e)$/T.B.W., and $r = 0.82$ and $r' = 0.91$ for π'_s versus $(Na_e + K_e)$/T.B.W.] suggest that Na_e, K_e and T.B.W. are the primary determinants of Na'_s. The standard deviations from regression for the parameters Na'_s, $(Na_e + K_e)$/T.B.W. and π'_s, $(Na_e + K_e)$/T.B.W. were 5.6 mEq. per liter and 10.2 mOsm. per liter, respectively (see Table IV). These variations from regression are partly, if not wholly, a reflection of methodological errors since the standard deviation of reproducibility of

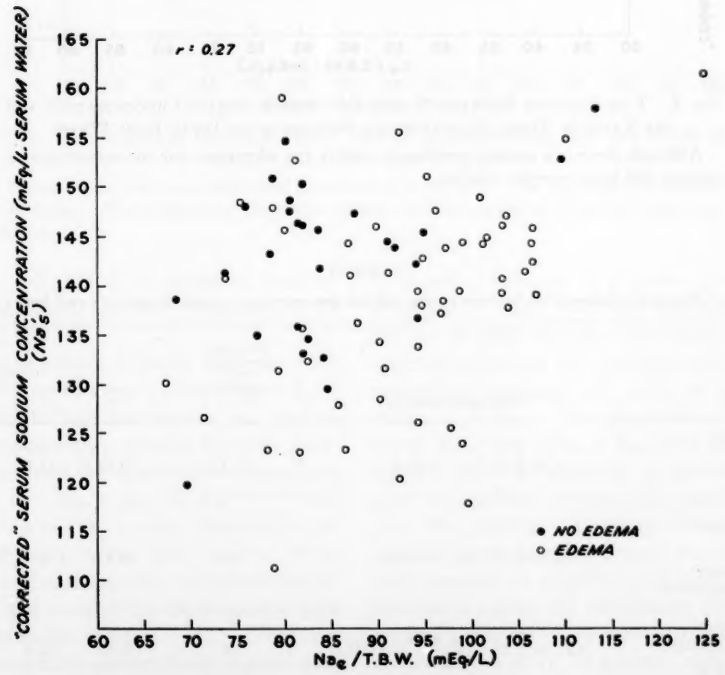

FIG. 5. THE RELATION BETWEEN "CORRECTED" SERUM SODIUM CONCENTRATION AND THE RATIO OF TOTAL EXCHANGEABLE SODIUM TO TOTAL BODY WATER

The correlation is quite limited for both edematous and nonedematous patients and a regression equation is not justified.

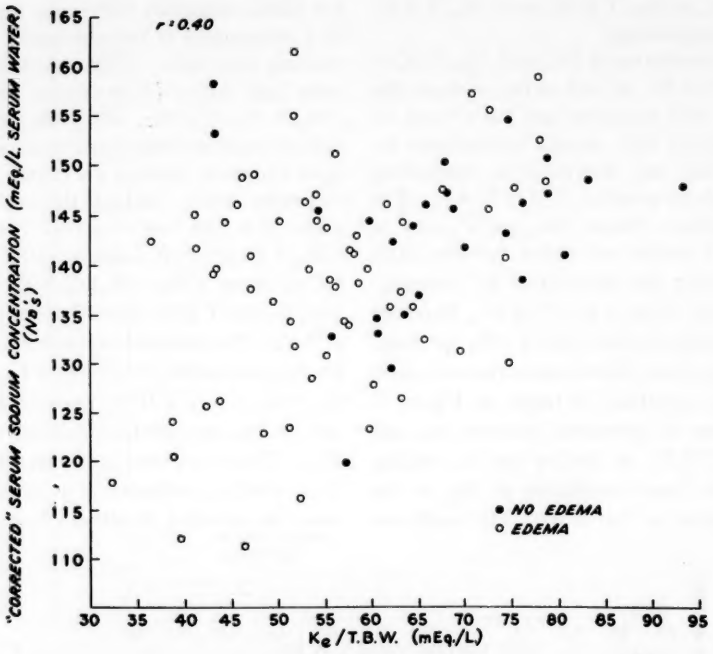

FIG. 6. THE RELATION BETWEEN "CORRECTED" SERUM SODIUM CONCENTRATION AND THE RATIO OF TOTAL EXCHANGEABLE POTASSIUM TO TOTAL BODY WATER

Although there is a modest correlation, neither the edematous nor the nonedematous patients fall into a precise relation.

TABLE IV

Summary of statistical relationships between serum sodium concentration, serum osmolarity and body composition

Parameters	Regression equation	Standard deviation from regression $S_{y.x}$	Correlation			
			r	t	p	r'
Serum osmolarity Serum sodium concentration	$\pi_s = 2.63\ Na_s - 65.4$	17.4	0.81	23.1	<0.001	0.82
"Corrected" serum osmolarity "Corrected" serum sodium concentration	$\pi'_s = 1.75\ Na'_s + 10.1$	5.6	0.97	121	<0.001	0.98
"Corrected" serum sodium concentration $(Na_e + K_e)$/T.B.W.	$Na'_s = 1.11 \frac{(Na_e + K_e)}{T.B.W.} - 25.6$	5.6	0.83	24.4	<0.001	0.92
"Corrected" serum osmolarity $(Na_e + K_e)$/T.B.W.	$\pi'_s = 2.09 \frac{(Na_e + K_e)}{T.B.W.} - 56.7$	10.2	0.82	19.8	<0.001	0.91

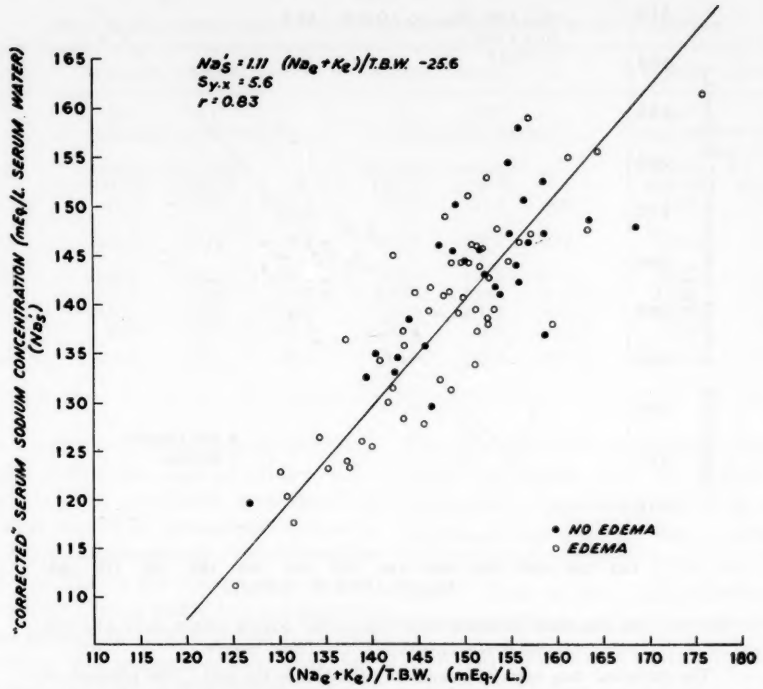

FIG. 7. THE RELATION BETWEEN SERUM SODIUM CONCENTRATION AND THE RATIO OF $(Na_e + K_e)$/TOTAL BODY WATER

The statistical data were calculated as described in the text. The regression is assumed to be linear. The presence or absence of edema does not appear to affect the regression relation.

the ratio $(Na_e + K_e)$/T.B.W. estimated from individual reproducibility of each component is 3.8 mEq. per liter.

The regression relation between Na'_s and $(Na_e + K_e)$/T.B.W. and between π'_s and $(Na_e + K_e)$/T.B.W., despite the high correlations, may include two or more populations. At least two sets of circumstances may alter the quantitative dependence of Na'_s or π'_s on $(Na_e + K_e)$/T.B.W.: *a*) acid-base disturbances and *b*) osmotic inequalities between cells and extracellular fluid (ECF). Bone sodium consists of exchangeable and nonexchangeable fractions, and most of the exchangeable bone sodium is osmotically inactive (15). Since acidosis will considerably reduce bone sodium content (17, 42), acid-base disturbances may shift the relationship between Na'_s and body composition. Figure 9 depicts the distribution of Na'_s versus $(Na_e + K_e)$/T.B.W. in subjects with arterial pH < 7.36 and in a second group with arterial pH > 7.48. Although the observations are too few to allow definite conclusions, there is no apparent deviation or variation from the general regression equation, and most of the data fall within ± 1 $S_{y.x}$. The quantitative relation between Na'_s and $(Na_e + K_e)$/T.B.W., therefore, appears to be independent of arterial pH. Osmotic inequalities among body fluids may influence this relation. Hyperglycemia particularly, and to a lesser extent azotemia, may be associated with transient or possibly even sustained osmotic differences across cell membranes (38, 41). Accordingly, Figure 10 shows the relation of Na'_s to $(Na_e + K_e)$/T.B.W. in patients with NPN > 50 mg. per 100 ml. of plasma or with glucose > 130 mg. per 100 ml. of plasma. There is no apparent deviation from the general regression equation, and no separation into different populations.

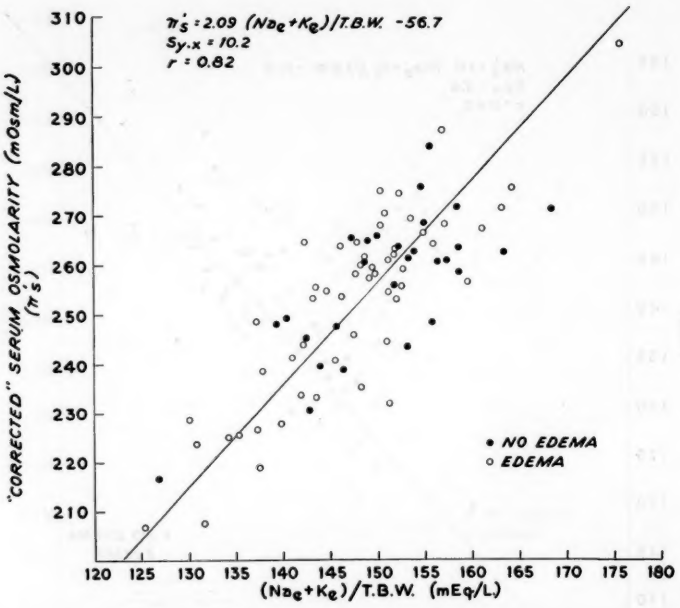

FIG. 8. THE RELATION BETWEEN THE "CORRECTED" SERUM OSMOLARITY AND THE RATIO OF $(Na_e + K_e)$/TOTAL BODY WATER

The statistical data were calculated as described in the text. The presence or absence of edema does not appear to affect the regression relation.

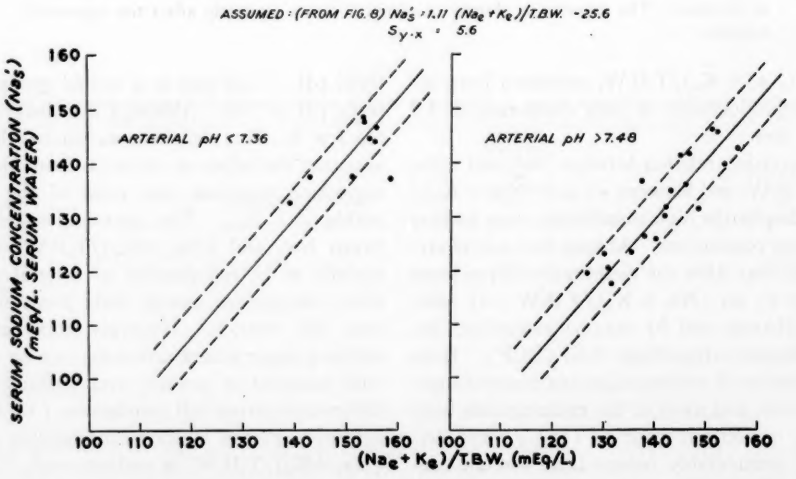

FIG. 9. THE RELATION OF Na'_s AND $(Na_e + K_e)$/TOTAL BODY WATER IN PATIENTS WITH ACIDOSIS (pH < 7.36) OR ALKALOSIS (pH > 7.48)

The regression line is taken from the relation depicted in Figure 8. Each broken line is one standard deviation from regression.

TABLE V

Serial studies of serum sodium concentration, serum osmolarity and body composition

	1st study	2nd study	Δ	1st study	2nd study	Δ	1st study	2nd study	Δ
Patient, *no.*	28	33		70	76		47a	53	
Diagnosis	chronic glomerulonephritis			panhypopituitarism			hypertension and congestive heart failure		
Clinical status	no edema	2+ edema		no edema	no edema		2+ edema	2+ edema	
Date	3/21/56	4/26/56		1/8/57	2/19/57		8/30/56	10/19/56	
Body weight, *Kg.*	47.6	51.1	+3.5	61.8	62.5	+0.7	48.6	49.8	+1.2
π'_s, *mOsm./L.* of serum water	261.6	207.0	−54.6	216.5	249.2	+32.7	256.4	208.0	−48.4
Na'_s, *mEq./L.* of serum water	144.0	111.2	−32.8	119.7	135.0	+15.3	138.5	117.6	−20.9
Na_e, *mEq.*	2,874	2,839	−35	2,497	2,645	+166	3,046	3,734	+688
K_e, *mEq.*	2,017	1,671	−346	2,039	2,180	+141	1,750	1,211	−539
T.B.W., *L.*	31.5	36.0	+4.5	35.6	34.4	−1.2	31.6	37.6	+6.0
$\frac{Na_e + K_e}{T.B.W.}$, *mEq./L.*	155.4	125.3	−30.1	126.9	140.3	+13.4	151.9	131.6	−20.3

The data summarized above indicate that Na'_s predominantly reflects the ratio of $(Na_e + K_e)$/T.B.W. and that this correlation is independent of differences in the pH of extracellular fluid or in glucose or NPN concentrations. The regression equation of this relation has a slope of 1.11 (see Table IV) and implies almost a 1:1 correspondence between changes in $(Na_e + K_e)$/T.B.W. and changes in Na'_s. The equation relating serial differences in Na'_s and $(Na_e + K_e)$/T.B.W. is:

$$Na_{s.1} - Na_{s.2} = 1.11\,[(Na_e + K_e)/T.B.W.]_1 - 1.11\,[(Na_e + K_e)/T.B.W.]_2$$

or,

$$\Delta Na'_s = 1.11\Delta[(Na_e + K_e)/T.B.W.]. \quad 7)$$

In three instances serial observations were made. The results, summarized in Table V, confirm the prediction of proportional changes between Na'_s and $(Na_e + K_e)$/T.B.W. independent of the presence or absence of edema. The proportionality to changes in π'_s is also obvious. These data confirm the report of Deming and Gerbode (11) on the synchronous changes in Na_s and sodium, potassium and water balance in patients after mitral valvulotomy and of Wynn (39) in similar studies. Wilson, Edelman, Brooks, Myrden, Harken and Moore (4) made serial observations of Na_s, Na_e, K_e and T.B.W. in patients before and after mitral valve surgery. When analyzed in terms of serial differences between successive observations, their data, combined with the values from Table V, demonstrate the same close correlation between ΔNa_s and $\Delta(Na_e + K_e)$/T.B.W. (see Figure 11). The slope, 0.83, and the intercept, 0.1, are in excellent agreement with the derived prediction (Equation 7), considering that in the earlier study (4) Na_s is expressed per liter of serum and that K_e was determined at 24 hours and T.B.W. at 3 hours. There is little doubt, therefore, that both among individuals and in any one individual, Na'_s is strongly dependent on $(Na_e + K_e)$/T.B.W.

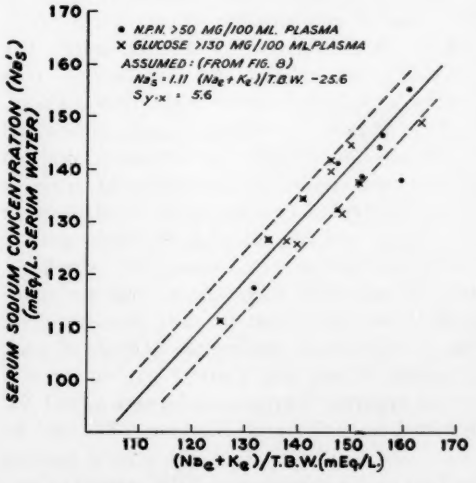

FIG. 10. THE RELATION OF Na'_s TO $(Na_e + K_e)$/TOTAL BODY WATER IN PATIENTS WITH AZOTEMIA OR HYPERGLYCEMIA

The regression line is taken from the relation depicted in Figure 8. Each broken line is one standard deviation from regression.

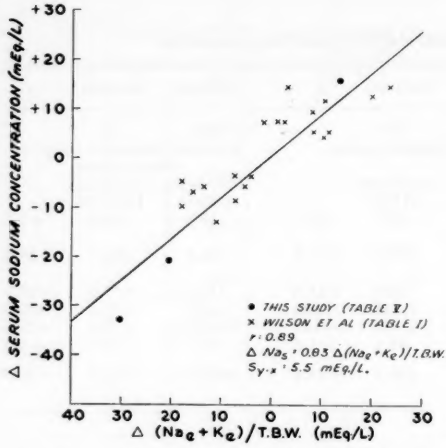

FIG. 11. SERIAL DIFFERENCES IN SERUM SODIUM CONCENTRATION AND THE RATIO $(Na_e + K_e)$/TOTAL BODY WATER

The data from Wilson and co-workers (4) are successive observations where the change in Na_s exceeded 3 mEq. per liter. The regression equation was calculated on the assumption of a linear relationship.

DISCUSSION

Figure 2 shows a linear regression of π'_s on Na'_s over a concentration range of Na'_s from 108.9 to 192.5 mEq. per liter of serum water. The slope, $\lambda = 1.75$, indicates that there is little or no binding of sodium in serum. Multivalent anions, such as phosphates, sulfates, organic anions and serum proteins, provide some electrochemical but lesser osmotic equivalence for sodium in serum, so that probably little or no difference exists between the activity and concentration of sodium salts in serum when referred to aqueous NaCl standards. Glucose and NPN contribute to total serum osmolarity in proportion to their concentrations. These observations confirm the findings of Leaf, Chatillon, Wrong and Tuttle (38), and account for the apparent discrepancies between π_s and Na_s reported by Talso and associates (34) and by Rubin and associates (36). The general equation relating π_s, Na_s, glucose and NPN appears to hold for the full range of clinical variations and without regard to the seriousness of illness (see under Results). The residual osmotic activity (~ 10 mOsm. per liter), which is given by the zero intercept of Equation 6, furthermore, can be reasonably assigned to the known amounts of other salts and trace substances in serum (37).

The data demonstrate poor correlations between Na'_s and Na_e, K_e, T.B.W., Na_e/T.B.W. and K_e/T.B.W. (see under Results and Figures 3 through 6). The linear correlation between Na'_s and $(Na_e + K_e)$/T.B.W. ($r = 0.83$, $r' = 0.92$) accounts for the reported effects of each of these components of body composition on Na_s (1–12, 14, 34, 38, 39). That Na'_s is a reflection of the ratio of the sum of exchangeable monovalent cation $(Na_e + K_e)$ to T.B.W. is confirmed by the approximately 1 : 1 correlation between sequential changes in Na'_s and $(Na_e + K_e)$/T.B.W. in the same individual (see Table V and Figure 11).

In 1944, Elkinton, Winkler and Danowski (43), using Na_s as the primary index of ECF osmolarity, tested the hypothesis of body fluid iso-osmolarity by comparing the sum of sodium balance (b.Na) and potassium balance (b.K) with the change in total osmotically active base. The expression they derived relating total base balance to changes in Na_s and in T.B.W. is:

$$(\text{b.Na} + \text{b.K}) = \text{T.B.W.}_1\,(0.95\,Na_{s,1} + 10) - \text{T.B.W.}_2\,(0.95\,Na_{s,2} + 10). \quad 8)$$

Despite very good correlations between observed and predicted base balances, the authors concluded that a significant and variable fraction of cell base is osmotically inactive. Deming and Gerbode (11) showed that the change in serum sodium concentration correlated closely with net change in sodium and potassium balance per unit change in body water. Recently, Wynn (39) compared the observed and predicted changes in serum cation concentration and found that the formulation of Elkinton and associates (43) predicts such changes within the limits of experimental error. These findings agree with ours since, assuming that sodium and potassium balance are equal to ΔNa_e and ΔK_e, Equation 8 is equivalent to Equation 7 except for minor details.

Variations in extracellular pH, glucose or NPN concentrations appear to have no influence on the character of the correlation between Na_s and Na_e, K_e and T.B.W. (see Figures 9 and 10). These data do not exclude the possibility that factors other than Na_e, K_e and T.B.W. alter Na_s. To demonstrate the existence of such factors, however, it would be necessary to show changes in

Na'_s independent of changes in the ratio $(Na_e + K_e)$/T.B.W. The concept of internal redistribution or "shifts" in electrolytes, so often invoked to explain changes in Na_s in disease states, needs to be re-examined since no evidence for the existence of such shifts without simultaneous changes in the amounts of sodium, potassium or water in the body is apparent in our data or in those referred to above (11, 39).

The relationships between π'_s, Na'_s and $(Na_e + K_e)$/T.B.W. are compatible with the concept of a passive distribution of water across cell membranes in proportion to solute activity. The possibility of parallel changes in intracellular and extracellular osmolarity with either a constant ratio or a constant difference between phases is not definitively excluded, but seems unlikely. Current evidence indicates that there are no sustained osmotic gradients across cell membranes, although transient gradients occur almost continuously as a result of cellular metabolic activity and absorption of ingested solute and water (8, 38, 39, 40, 44–47). The influence of potassium on Na_s is presumably a reflection of its contribution to intracellular osmolarity. The zero intercept of the regression equation for Na'_s versus $(Na_e + K_e)$/T.B.W. is a negative constant ($-$ 25.6 mEq. per liter), which probably is a measure of the quantity of osmotically inactive exchangeable sodium and potassium per unit of body water. Since body water in this group of subjects averaged 36.1 liters (see Table III), the estimated osmotically inactive Na + K is approximately 920 mEq. Osmotically inactive exchangeable bone sodium, which is about 750 mEq., probably accounts for almost all of this quantity (15). The calculated ratio of osmotically inactive to total exchangeable potassium, therefore, is less than 10 per cent (*i.e.*, $\sim$ 170/2099), which suggests that there is little or no discrepancy between exchangeable and osmotically active potassium. However, this calculation is valid only if Na_s and $(Na_e + K_e)$/T.B.W. are linearly related over the entire range of their possible values.

Figures 7, 9 and 10 indicate that Na'_s reflects the proportion of $(Na_e + K_e)$ to T.B.W. This observation provides a rational basis for the classification of hyponatremic and hypernatremic states. Hyponatremia may reflect either *a*) primary sodium deficit, *b*) primary potassium deficit, *c*) primary water excess or *d*) combinations of these. Conversely, hypernatremia may reflect either *a*) primary sodium excess, *b*) primary potassium excess, *c*) primary water deficit or *d*) combinations of these. Hyponatremia in patients with gastrointestinal fluid loss probably results from loss of sodium and potassium (16, 48, 49). Hyponatremia in edematous subjects, on the other hand, appears to be a consequence of the loss of potassium and gain of water, as exemplified by Patients 28 and 47a in Table V. This pattern is also prominent in postoperative hyponatremia; in all but 2 of the 11 patients studied by Wilson and associates, (4) the postoperative fall in Na_s was associated with a rise in Na_e, a fall in K_e, and a rise in T.B.W. Hypernatremia has not yet been studied in terms of these components of body composition, but relative or absolute body water depletion is probably its most frequent cause. Diabetes insipidus and water deprivation are two classical examples of this state. Primary excess of potassium is probably not involved in the pathogenesis of hypernatremia, inasmuch as no instance of an abnormally high body potassium content as a consequence of disease has been reported (1, 18). However, reversal of hyponatremia by potassium-loading in patients who presumably were potassium-depleted has been observed (12), and this strategem promises to be valuable in the treatment of this disturbance in edematous subjects (7).

The correction of the hyponatremia presumed to result from sodium depletion is usually based on the formula:

$$\text{Na deficit} = (140 - Na_s)\text{T.B.W.} \qquad 9)$$

This equation, which is supported by clinical experience, is based on the assumption that isotonic conditions prevail throughout body water (7). Wolf and McDowell (40) emphasized that this formula assumes that T.B.W. is unchanged by therapy and revised Equation 9 to include the effect of changes in T.B.W.

$$\text{Na deficit} = (140 - Na_s)\,\text{T.B.W.}_1 + (\Delta H_2O)\,140, \qquad 10)$$

where $\text{T.B.W.}_2 - \text{T.B.W.}_1 = \Delta H_2O$. By substituting and rearranging terms their equation may

be expressed:

$$\text{Na deficit} = 140\,\text{T.B.W.}_{.2} - \text{Na}_s\,\text{T.B.W.}_{.1}. \quad (11)$$

Both Equations 9 and 10 are special cases of the general regression equation derived in Figure 8. There are, in fact, three equations which may be derived from the general relation between Na_s and body composition (see Equation 7). This equation can be simplified if it is assumed that either *a*) T.B.W. is unchanged by therapy ($\text{T.B.W.}_{.c}$) or *b*) K_e is unchanged by therapy ($K_{e.c}$), or *c*) both T.B.W. and K_e remain constant. These three assumptions each lead to separate modifications of Equation 7. The general relation expressed in Equation 7 may be written:

$$\text{Na}_{s.2} - \text{Na}_{s.1} = 1.11 \times \left[\frac{\text{Na}_{e.2} + \text{K}_{e.2}}{\text{T.B.W.}_{.2}} - \frac{\text{Na}_{e.1} + \text{K}_{e.1}}{\text{T.B.W.}_{.1}}\right]$$

a) For the special case where T.B.W. is constant and both Na_e and K_e change as a result of therapy, the equation becomes:

$$\text{Na}_{e.2} - \text{Na}_{e.1} = [140 - \text{Na}_{s.1}]\text{T.B.W.}/1.11 - [\text{K}_{e.2} - \text{K}_{e.1}]. \quad (12)$$

b) For the special case where K_e is constant but Na_e and T.B.W. are altered by therapy:

$$\text{Na}_{e.2} - \text{Na}_{e.1} = [140\,\text{T.B.W.}_{.2} - \text{Na}_{s.1}\,\text{T.B.W.}_{.1} + \Delta\text{H}_2\text{O}\cdot 25.6]\,1/11. \quad (13)$$

c) Finally, for the special case where only Na_e is variable but $K_{e.2} = K_{e.1}$ and $\text{T.B.W.}_{.2} = \text{T.B.W.}_{.1}$:

$$\text{Na}_{e.2} - \text{Na}_{e.1} - (140 - \text{Na}_{s.1})\,\text{T.B.W.}_e/1.11. \quad (14)$$

Equations 14 and 9 are identical, except for the factor 1/1.11, if the sodium deficit is equated with ΔNa_e. The Wolf-McDowell formulation, Equation 11, is similar to Equation 13, except for the term $\Delta H_2O \cdot 25.6$, which appears as a consequence of the difference between total exchangeable cation and total osmotically active cation. A similar set of equations may also be derived from Equation 8. Our data, therefore, provide an experimental basis for the formulas used in clinical manipulations and demonstrate the coordinated influence of the three major components of body composition on serum sodium concentration.

SUMMARY

The relationships between serum sodium concentration (Na_s), serum osmolarity (π_s), total exchangeable sodium (Na_e), total exchangeable potassium (K_e) and total body water (T.B.W.) were explored by simultaneous measurements in a heterogeneous group of chronically ill patients.

Serum osmolarity correlates closely with serum sodium concentration expressed as mEq. per liter of serum water (Na'_s) when appropriate corrections are made for the osmotic contributions of glucose and nonprotein nitrogen (NPN). Serum sodium concentration is only partially or poorly correlated with total exchangeable sodium/body weight, total exchangeable potassium/body weight, total body water/body weight, total exchangeable sodium/total body water and total exchangeable potassium/total body water. A high degree of correlation, however, exists between serum sodium concentration (Na'_s) or "corrected" serum osmolarity (π'_s) and the ratio $(Na_e + K_e)$/T.B.W. This relation is confirmed by the high correlation between the simultaneous serial differences in Na'_s and $(Na_e + K_e)$/T.B.W. The regression of Na'_s on this ratio appears to be independent of arterial pH, plasma glucose or plasma NPN concentrations.

The implications of these data with respect to osmotic gradients in various components of body water and to evaluation of osmotic activity of body potassium are explored. Body water appears to be passively distributed in proportion to osmotic activity, and all or almost all of body potassium is osmotically active.

A classification scheme for hyponatremia and hypernatremia is presented, and equations for estimating sodium deficits are derived.

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SURVIVAL OF BLOOD PLATELETS LABELED WITH CHROMIUM[51] [1, 2]

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Various criteria exist for the evaluation of the integrity, viability and physiological activity of human blood platelets. Alterations in platelet morphology, clot promoting and clot retracting activities, oxygen consumption, clinical effect on bleeding disorders, and the life span of platelets in thrombocytopenic recipients have been used as measurements of platelet function. The transfusion studies in thrombocytopenic recipients indicate that platelets have a life span of three to seven days and have been helpful in the classification of certain pathological disorders (2). These observations have been dependent upon obtaining donors with high platelet counts and accepting the errors associated with platelet counting. The difficulty in procuring adequate donors and the desirability of measurements other than platelet enumeration have initiated numerous studies of the *in vivo* survival of platelets labeled with radioactive isotopes.

The earlier studies concerning measurement of the life span of blood platelets with isotopes prior to 1955 have been reviewed by Odell, Tausche and Furth (3). In Table I the various isotope techniques previously reported are tabulated for comparison. The recent report by Leeksma and Cohen (13) has demonstrated a life span of labeled platelets of eight to nine days. These investigators followed the activity of radioactive phosphorus in platelets after the recipient had received diisopropylfluorophosphonate (DFP^{32}) parenterally. Reisner, Keating, Friesen and Loeffler (14) have noted that radioactive sodium chromate could be bound to platelets *in vitro*. When these platelets were transfused to recipients, a survival period of five to eight days could be measured. Similarly, transfusions of *in vivo* labeled P^{32} platelets from polycythemic donors have had a life span of seven days in normal recipients (15).

The present investigation was in progress at the time of the observations by Reisner and co-workers (14). A workable technique is presented for the *in vitro* labeling of human blood platelets with radioactive chromium and evaluating their survival after transfusion to normal recipients. Comments regarding details of the method will be presented.

METHODS

Material. Whole blood donations were obtained from volunteer medical staff and patients. In many instances *in vitro* studies were done using blood obtained from therapeutic phlebotomies.

Preparation of platelet suspension. The following technique was derived from past experience in the preparation of platelet suspensions using plastic bag equipment. Five hundred ml. of whole blood is collected by gravity into polyvinyl chloride plastic bags (Fenwal Laboratories) containing 50 ml. of 1.5 per cent disodium ethylenediaminetetracetic dihydrate (Na_2EDTA) in saline. As noted previously (16) Arquad® [4]-coated 15 gauge malehub needles are used. The plastic tubing leading from the needle to the plastic bag has a "Y" type division that allows 10 ml. of blood to be withdrawn to exclude tissue juice contamination and to evaluate venous blood flow. The whole blood is centrifuged at 2° C. for 15 minutes at 1,300 rpm. (325 G) in an International Centrifuge, Model PR-2. After centrifugation, the platelet-rich plasma is transferred to another plastic bag containing 15 ml. of 2 per cent Triton® [5] in saline using a piercing

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[2] A preliminary report was presented at The Sixth Congress of The International Society of Blood Transfusion, 1956 (1).

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[4] Arquad® is a trade-mark of Armour Chemical Division, Armour and Co., Chicago, for Tris (2-hydroxyethyl) dodecylamine and N,N'(2,3-dihydroxypropyl)-N-alkyl-trimethylenediamine (A-15).

[5] Triton® is a trade-mark of Rohm & Haas Co., Philadelphia, for oxyethylated tertiary octyl phenol formalde-

TABLE I

Summary of isotope platelet survival studies

Author	Year	Isotope	Mode of tagging	Species	Indicated life span
Julliard and associates (4)	1952	P^{32}	*In vitro*	Human	2 hours
Mueller (5)	1952	P^{32}	*In vitro*	Rabbit	2 hours
Odell and associates (6)	1953	C^{14}	*In vivo*	Rat	3–4 days
Morgan and associates (7)	1954	I^{131}	*In vitro*	Rabbit	1–2 hours
Robertson and associates (8)	1954	$Cr^{51}Cl_3$	*In vitro*	Rat	2 days
Odell and associates (9)	1954	$C^{14} + S^{35}$	*In vivo*	Rat	5–6 days
Odell and associates (10)	1955	C^{14}	*In vitro*	Rat	1–3 days
Odell and associates (10)	1955	S^{35}	*In vivo*	Rat	4–5 days
Desai and associates (11)	1955	P^{32}	*In vitro*	Human	Apparent ½-survival time 35–50 hours
Morgan and associates (12)	1955	$Na_2Cr^{51}O_4$	*In vitro*	Rabbit	3–4 days
Leeksma and Cohen (13)	1956	DFP^{32}	*In vivo*	Human	8–9 days
Reisner and associates (14)	1956	$Na_2Cr^{51}O_4$	*In vitro*	Human	5–8 days
Adelson and associates (15)	1957	P^{32}	*In vivo*	Human	7 days

coupler (16). A positive pressure pneumatic press is helpful in allowing careful separation of plasma from the red cells and buffy coat. The plastic bag containing the platelet-rich plasma is distended with air to prevent platelet trapping in folds of the bag during centrifugation and is then centrifuged at 2° C. for 30 minutes at 2,300 rpm. (1,000 G). The platelets separate out as a creamy button at the bottom of the bag. The platelet-poor supernatant plasma is withdrawn under sterile conditions and saved, leaving approximately 10 ml. of residual plasma with the platelet button.

Labeling of platelets. Through the plastic inlet tubing attached to the bag, 10 ml. of 0.2 per cent Triton® in saline solution is injected by syringe. The bag is kneaded between the fingers to resuspend the platelet button until no macroscopic clumps are visible. Three hundred microcuries of sterile radioactive sodium chromate with a specific activity of 20 microcuries Cr^{51} per microgram is injected into the bag through the plastic tubing and directed into the platelet suspension. The chromate solution is mixed and the platelet suspension is incubated for 15 minutes at room temperature (20 to 22° C.). Thereafter, the suspension is centrifuged again at 2° C. for 30 minutes at 2,300 rpm. (1,000 G), and the plasma-Triton® solution is drained from the platelet button by gravity and saved to determine its residual Cr^{51} activity. Fifteen ml. of the sterile platelet-poor plasma, previously saved, and 15 ml. of 0.2 per cent Triton®-saline solution are added to the platelet button and the platelets resuspended. One hundred milligrams of a 5 per cent ascorbic acid solution is then added to the platelet suspension to prevent red cell binding in the recipient by excess $Na_2Cr^{51}O_4$. The platelets are dispersed by kneading, and about 100 ml. of air is allowed to enter the bag to facilitate complete infusion of the platelet suspension. A short piece of plastic tubing is attached to the plastic bag using a piercing coupler to decrease loss of platelets adhering to the tubing. Usually a saline infusion has been started in the recipient to assure the rapid injection of the 30 to 35 ml. volume of platelet suspension. From the average donor, the platelet concentrate in a 35 ml. volume usually has a 3.5×10^6 cu. mm. platelet count. The entire procedure requires about two hours from the phlebotomy to the infusion of labeled platelets.

hyde polymer and supplied as entsufon (WR-1339) by Winthrop Laboratories, New York.

Comments on method

1. Anticoagulant. Plasma preparations prepared with acid citrate dextrose (ACD) solution (National Institutes of Health formula) have numerous microscopic aggregations of platelets that do not resuspend after centrifugation and labeling with Cr^{51}. Earlier studies have demonstrated that platelets are discrete and not clumped when Na_2EDTA was used as the anticoagulant (16, 17). No evaluation was made in this present study to determine if microscopic clumping of platelets influenced viability after labeling and infusion.

2. Differential centrifugation. The speeds listed have been derived by arbitrary studies to achieve the maximum yield of platelets in platelet-rich plasma (16). About 70 per cent of the platelets are recovered from whole blood by this procedure.

3. Platelet suspension. Irreversible platelet agglutination has been controlled by the use of surface-active agents, especially Triton® (WR-1339) (18), which allows rapid suspension of platelets and is an important factor in decreasing the time interval of handling platelets *in vitro*.

Platelet radioactivity in the recipient. To follow the fate of the transfused Cr^{51} labeled platelets in the recipient, 20 ml. blood samples are drawn from the recipient at one-half, two and four hours, and daily thereafter, until no further radioactivity can be detected in the recipient's platelets. The blood is collected using Arquad®-coated needles and silicone-coated syringes, and is placed in 50 ml. silicone-coated tubes containing 2.0 ml. of 3 per cent Na_2EDTA solution in saline. Two ml. of

whole blood is removed to determine radioactivity of the whole blood sample. The tube is then centrifuged at 2° C. for 15 minutes at 1,000 rpm (175 G). The platelet-rich plasma is removed from the red cells with a silicone-coated pipette and transferred to a 10 ml. silicone-coated test tube which is centrifuged at 3,000 rpm (1,200 G) for 30 minutes at 2° C. The platelet-poor plasma is decanted and the platelet "button" at the bottom of the tube is resuspended in 10 ml. of 0.2 per cent Triton® saline solution and recentrifuged. The supernatant saline solution is decanted and saved to determine its radioactivity, while the platelet button remains in the test tube for determination of its radioactivity. The red cell mass and buffy coat from the initial centrifugation are eluted twice with 10 ml. aliquots of 0.2 per cent Triton® saline solution. The supernatant saline solutions from the two centrifugations are pooled and saved to determine radioactivity. Platelet counts of the whole blood, platelet-rich plasma, platelet-poor plasma, red cell mass and red cell eluates ("wash solution") are determined by phase contrast microscopy (19). Two ml. aliquots of whole blood, platelet-poor plasma, platelet "wash solution," red cell mass and red cell "wash solution" are used to determine radioactivity in these respective separations.

Daily 24 hour urine collections were obtained from four recipients for 22 days to determine excretion of Cr^{51} activity. These specimens are counted in a thallium-activated sodium iodide well-type scintillation counter (Tracerlab Model P-20A) with three-inch lead shielding. All samples are counted to maintain a counting error of less than 5 per cent. Values followed for more than 24 hours are corrected for physical decay.

Determination of Cr^{51} labeling of platelets. One ml. of the labeled platelet suspension is withdrawn from the plastic bag before infusion to determine the total radioactivity of the volume transfused. One ml. of the plasma-Triton® solution is removed before suspension of the labeled platelets in the platelet-poor plasma and is measured to determine the radioactivity remaining in plasma-Triton® solution that was not bound to platelets. The summation of the radioactivity is considered to be the total Cr^{51} that was available to label the platelets from the 300 microcuries injected into the bag. Inasmuch as some platelets will adhere to the plastic bag surface, the injected labeling dose of chromate (300 microcuries) is not used to determine the percentage of activity bound to the platelets.

$$\frac{\text{Radioactivity of final platelet suspension}}{\text{Radioactivity platelet-poor plasma} + \text{Radioactivity of final platelet suspension}} \times 100 = \text{per cent } Cr^{51}\text{-labeled platelets}$$

Previous studies have indicated that one may anticipate by platelet counting 50 to 60 per cent initial recovery of the transfused concentrated platelet suspension in the recipient's blood volume (16). These studies involved the platelet suspension in only one rapid centrifugation (1,000 G). The present method of labeling involves centrifuging the platelets twice at high speed. A single platelet recovery study has been done without Cr^{51} labeling to evaluate such a manipulation of the platelets. In this instance 46 per cent of the theoretical platelet yield was counted in a thrombocytopenic recipient. The 50 per cent loss of the transfused platelets has been attributed to viability changes during manipulation.

In vitro studies. To determine the optimal conditions for labeling platelets with radioactive chromium, numerous *in vitro* observations were performed. Whole blood was collected in 500 and 125 ml. plastic bags and centrifuged to prepare the platelet suspensions as described previously. Platelet suspensions in 10 ml. of plasma were incubated with radioactive sodium chromate at 3° C., 20 to 22° C. (room temperature), and 37° C. The suspensions were centrifuged and the residual radioactivity of the platelets was expressed as the percentage of the total radioactivity of the platelet suspension before centrifugation. Approximately 1 per cent of the Cr^{51} activity remained with the platelet mass at 3° C. and 37° C., while 3.6 per cent remained at 22° C.

The lower percentage at 37° C. has been attributed to elution of the radioactivity into the surrounding plasma media. Likewise, the Cr^{51} bound to the platelets was not increased beyond an incubation period of 15 minutes. Labeled platelet preparations used for clinical studies were incubated for 15 minutes at room temperature.

Relationship of Cr^{51} concentration. To determine the efficiency of binding Cr^{51} to platelets, a platelet-plasma suspension was divided into 12 ml. aliquots in silicone-coated test tubes. The platelet count was 323,000 cu. mm. Varying amounts of $Na_2Cr^{51}O_4$ from 3 microcuries to 25 microcuries diluted to equal volumes in saline were added to the platelet suspension, and the samples were incubated 15 minutes at room temperature. The tubes were then centrifuged at 1,000 G at 3° C. and the platelet button measured for radioactivity. A linear relationship between the amount of Cr^{51} introduced into the platelet suspension and the radioactivity bound to the platelets was observed. Similarly, if the same experiment is repeated with the $Na_2Cr^{51}O_4$ remaining constant and the platelet count varied by plasma dilution, more radioactivity is found in the platelet button with the highest platelet count. In essence, the platelets will be labeled with Cr^{51} in direct relationship to the number of platelets in the suspension.

Suspending medium. When platelet-rich plasma is utilized as the medium in which to label platelets, 1 to 3 per cent of the total radioactive sodium chromate is bound to the platelets. Such suspensions usually had platelet counts of 300,000 cu. mm. Platelet concentrates suspended in 10 ml. of plasma (platelet count 2 million cu. mm.) averaged 9 per cent labeling, and when such concentrates were prepared from donor patients with polycythemia vera (platelet counts 5 to 6 million cu. mm.), 15 to 25 per cent of the radioactive chromate was bound to the platelets. The average platelet concentrate suspended in a saline-Triton® solution had 15 per cent of the radioactivity bound to the platelets.

Previous studies have emphasized the selective binding of chromic chloride to plasma proteins (20). Less than 1 per cent of Cr^{51} is bound to the platelets if $Cr^{51}Cl_3$ is used in platelet-rich plasma suspensions while platelet concentrates in 10 ml. of plasma bound 1 to 2 per cent of $Cr^{51}Cl_3$. Higher residual radioactivity in the platelet concentrate is obtained by the removal of plasma and suspension of platelets in saline-Triton® solution. However, use of saline-Triton® solution requires additional centrifugation of the platelets and a prolonged time of preparation. Likewise, phase microscopy studies reveal marked morphological changes in platelets suspended in saline. Therefore, to hasten the preparation of platelets for *in vivo* studies and to prevent effects of saline contact, the smaller percentage of bound Cr^{51} activity in plasma concentrates has been preferred in these studies.

Cr^{51} elution from platelets. The addition of ascorbic acid to the $Na_2Cr^{51}O_4$ labeled platelet concentrate reduces the chromium to trivalent form. *In vitro* incubation of the ascorbic acid-treated labeled platelets with red cells demonstrated no transfer of Cr^{51} to the red cells, and similar platelet suspensions do not label buffy coat preparations. One hour incubation at room temperature of the Cr^{51} labeled platelets in 40 ml. of platelet-poor plasma results in elution of 40 to 70 per cent of the radioactivity into the plasma, and ascorbic acid does not alter this rate of elution. Cr^{51} activity from the $Na_2Cr^{51}O_4$ labeled platelets is eluted to a similar degree in saline-Triton® solution, but platelets labeled with $Cr^{51}Cl_3$ have less elution (20 to 25 per cent) when incubated in plasma or saline. This rapid elution of Cr^{51} from the platelets has not been observed after infusion and may reflect improvement *in vivo* of platelet viability and metabolic function.

The release of Cr^{51} from the platelet membrane should not be confused with the binding of chromium to the plastic material. When $Cr^{51}Cl_3$ saline solution was introduced into a plastic bag with no platelets, 12 per cent of the injected radioactivity remained on the plastic surface even after repeated washing. Depending upon the length of the inlet tubing used to introduce the $Cr^{51}Cl_3$ into the bag, as much as 21 per cent of the injected dose may bind to the tubing and not be eluted despite repeated washing. In contrast $Na_2Cr^{51}O_4$ is associated with negligible adherence of radioactivity to plastic; namely, 0.1 per cent in the plastic bags and 0.04 per cent in the plastic inlet tubing. Similar observations have been noted in other applications of chromium salts (21).

It may be concluded from these *in vitro* observations that chromium[51] chloride and sodium chromate[51] can be used to label platelets. Sodium chromate offers greater ease in the preparation of the labeled platelets and less difficulty in the evaluation and measurement of radioactivity.

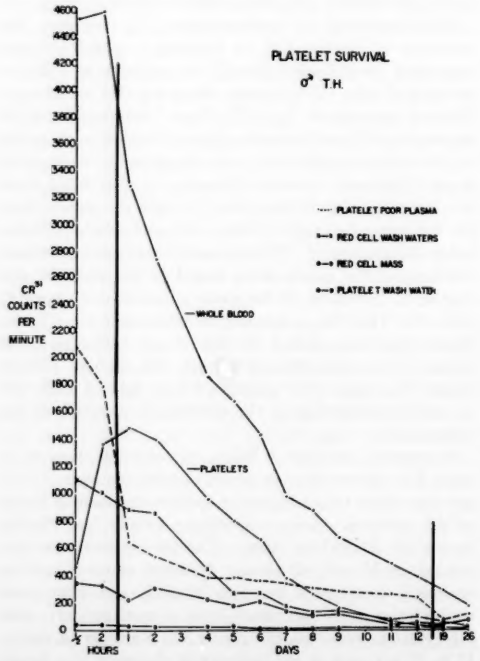

FIG. 1. MEASUREMENTS OF Cr^{51} ACTIVITY IN $Na_2Cr^{51}O_4$ LABELED PLATELETS AND SEPARATED BLOOD COMPONENTS AFTER INFUSION IN A NORMAL VOLUNTEER RECIPIENT

RESULTS

In vivo observations

Radioactive sodium chromate labeled platelets were transfused to 30 recipients with normal platelet counts. Heterologous or autologous labeled platelets were used with no untoward reactions such as pain, fever, urticaria or phlebitis. Inasmuch as each volunteer recipient received only one labeled platelet transfusion, there was no concern that platelet sensitization would develop to alter the survival period (2). In these studies all radioactive components of the recipient's venous blood samples were followed to evaluate localization of radioactivity. The term "wash water" was used to denote the saline washings of red cells and platelets, and the radioactivity of these components is graphed in Figure 1. The peak of the Cr^{51} activity of the platelet "button" at 24 hours after infusion is about 30 to 50 per cent of the whole blood radioactivity. The separated platelet button in this study indicates that the labeled platelets had a life span of 10 to 11 days. A parallel decline in whole blood activity is associated with the loss of platelet activity. The radioactivity of the separated platelet-poor plasma represents: *1*) un-

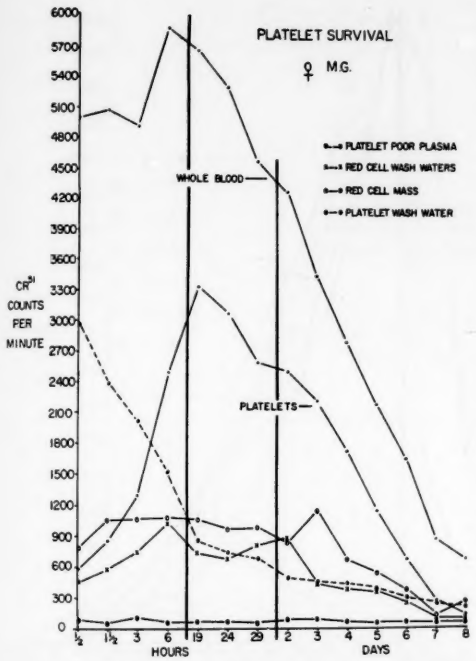

FIG. 2. $Na_2Cr^{51}O_4$ LABELED PLATELETS IN A NORMAL VOLUNTEER RECIPIENT

The maximum Cr^{51} activity was observed at 19 hours after infusion.

bound Cr^{51} in the plasma of the platelet concentrate that was not removed completely before the labeled platelets were resuspended in sterile platelet-poor plasma for infusion; and *2*) Cr^{51} from nonviable platelets that constitute the 50 per cent of platelets not accounted for in the recipient after infusion. The radioactivity of the platelet-poor plasma declines rapidly during the first 24 hours after infusion, although some Cr^{51} may be found in the plasma for a month indicating minimal labeling of plasma proteins by $Na_2Cr^{51}O_4$. The red cells are not tagged in this procedure aside from the few that remain in the platelet-rich plasma during preparation. The radioactivity in the red cell mass during the first 24 hours after the transfusion is derived from entrapped plasma. Thereafter the small residual radioactivity in the centrifuged red cells is due to platelets. The radioactivity in the saline washings (wash water) of the platelet button is negligible, about 1 per cent of the whole blood radioactivity.

The maximum radioactivity of the separated labeled platelets is always noted about 24 hours after the infusion. The platelet radioactivity was followed at frequent time intervals in one recipient to determine the maximum radioactivity which in this instance occurred at 19 hours (Figure 2). Probably the time intervals vary among recipients depending upon when the maximum numbers of labeled platelets reappear in the circulation. The maximum platelet activity observed 24 hours after infusion has been used to compare the per cent of labeled platelets appearing in the circulation during the first few hours. This progressive increase in Cr^{51} activity of the platelet "button" has been graphed in Figure 3. The marked variability of platelet activity half an hour after transfusion is startling. The delayed appearance of circulating platelets after transfusion of platelet concentrates into thrombocytopenic recipients has been noted previously (15, 16), (Figure 9).

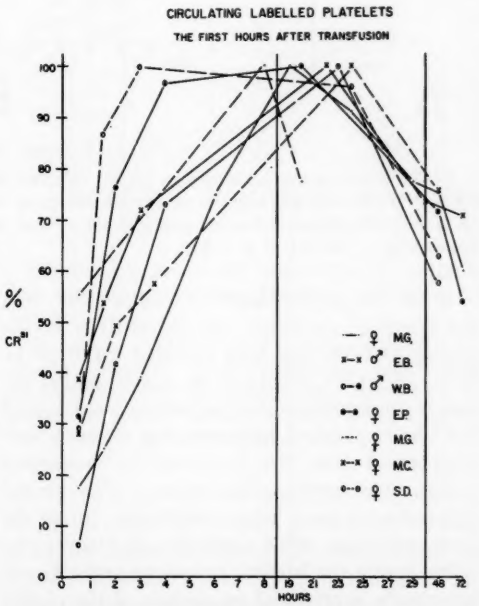

FIG. 3. PLATELET RADIOACTIVITY WITH TIME

The observed maximum Cr^{51} activity of the separated platelets is plotted as the 100 per cent value. The percentage of Cr^{51} during the initial period after infusion is related to the maximum value which is observed about 20 hours later.

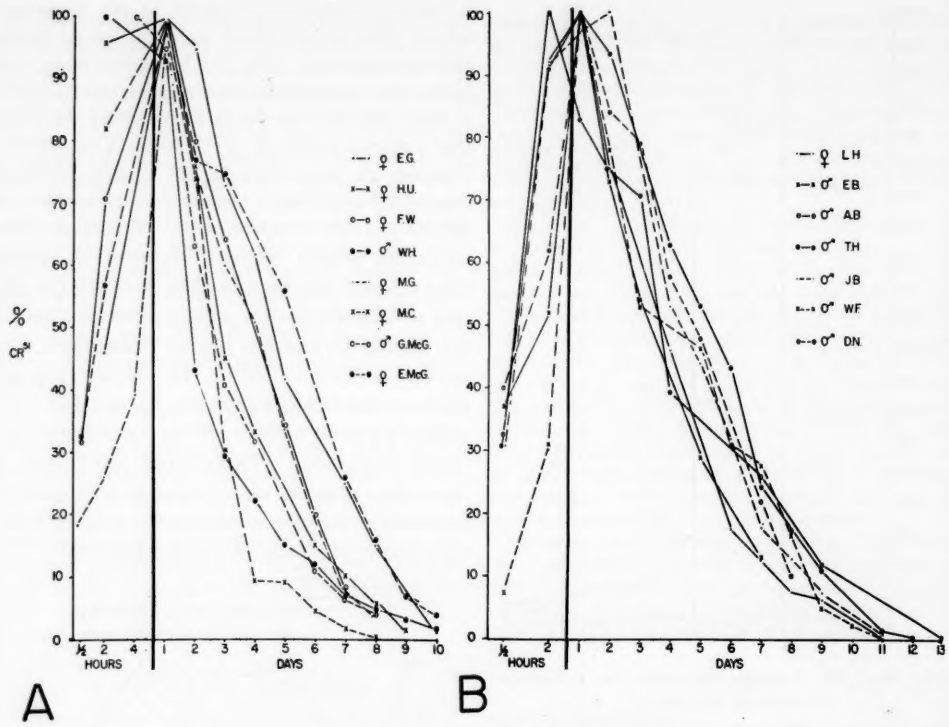

FIG. 4. PLATELET SURVIVAL IN NORMALS

A. The initial studies are shown in normal volunteer recipients by the technique described. The measurement of the Cr^{51} labeled platelets suggests an exponential curve of the platelet survival *in vivo*. B. The last seven studies performed with technical improvement indicate a linear loss of the labeled platelets from the circulation. Platelet radioactivity is plotted as in Figure 3.

In all the platelet survival studies done with the described procedure, the radioactivity of the labeled platelets has been followed for 9 to 11 days. The Cr^{51} activity of the platelet button observed during this period suggests a linear loss of the labeled platelets, indicating that platelets have disappeared from the circulation by senescence rather than random destruction. The plotted values have a more linear relationship during the first eight days, while alterations in platelet viability during the labeling procedure probably enhanced the exponential appearance of the plotted curves during the last three days that radioactivity was measured. The influence of the manipulations of the initial platelet concentrate may be appreciated by comparison of the survival curves in Figure 4. The eight platelet curves to the left represent values obtained during the early phase of the investigation, while the seven curves to the right represent the last seven recipients studied. The more linear loss of Cr^{51} platelet activity in the later studies reflects improved handling of the platelets during the labeling procedure.

Modifications of transfusion procedure

Previous studies have emphasized the value of ascorbic acid to prevent further binding of sodium chromate to the red cell membrane. One study was performed to determine whether ascorbic acid could be omitted from the technique (Figure 5). While the platelet button radioactivity could be measured, the Cr^{51} activity of the whole blood was 10 times as high as anticipated due to the labeling of the red cells *per se*. In certain patho-

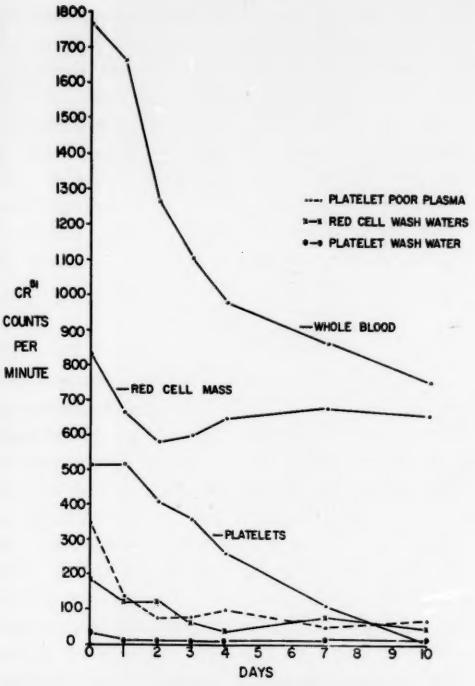

FIG. 5. PLATELET SURVIVAL WITHOUT ASCORBIC ACID—FEMALE, M. W.

The omission of ascorbic acid from the platelet concentrate before infusion does not influence the interpretation of the labeled platelet survival period. The labeling of red cells by the hexavalent chromium prolongs the period of circulating radioactivity and limits repeated observations in the recipient.

logical states the Cr^{51} bound to red cells would be cumulative if repeated observations were made to evaluate therapeutic responses in thrombocytopenic recipients.

In the present procedure the platelets are centrifuged after the initial labeling with $Na_2Cr^{51}O_4$ to remove residual Cr^{51} in the surrounding plasma. About 80 to 90 per cent of the Cr^{51} mixed with the platelet concentrate remains in the plasma. Hence it is desirable to remove the 240 to 270 microcuries of Cr^{51} from the suspension. However, this additional centrifugation may lessen platelet viability. Therefore, one study was done to determine whether omission of the last centrifugation in the preparation of the platelets for infusion would alter the observed survival period.

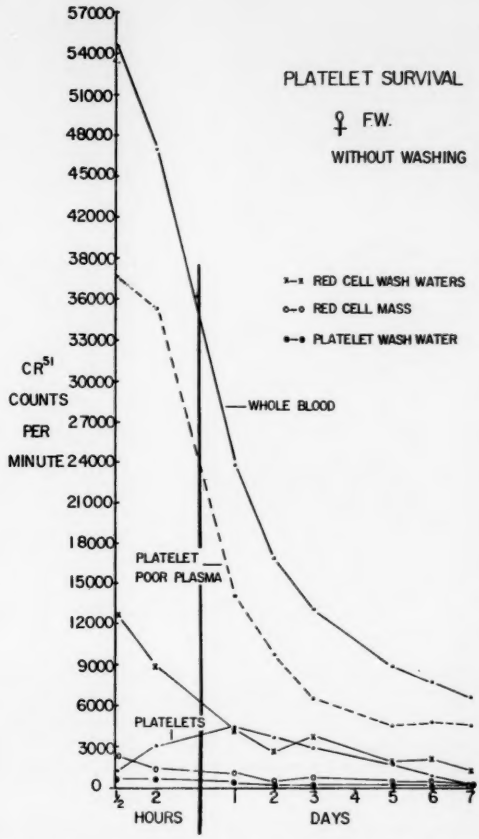

FIG. 6. INFUSION OF Cr^{51} LABELED PLATELETS WITHOUT REMOVAL OF EXCESS $Na_2Cr^{51}O_4$ IN PLASMA BY CENTRIFUGATION

There is no improvement or alteration of platelet values by elimination of this additional centrifugation. Measurements were discontinued after seven days, although adequate Cr^{51} activity was present in the platelet button for a longer period of observation.

In Figure 6 the survival period indicates no prolongation of the Cr^{51} platelet activity, and furthermore the higher dosage of Cr^{51} would limit the evaluation of platelets to one study and would not allow repeated observations in thrombocytopenic recipients.

The *in vitro* studies with $Cr^{51}Cl_3$ indicate that platelet concentrates could be labeled although the larger portion of radioactivity resides in the plasma of the final platelet concentrate. One

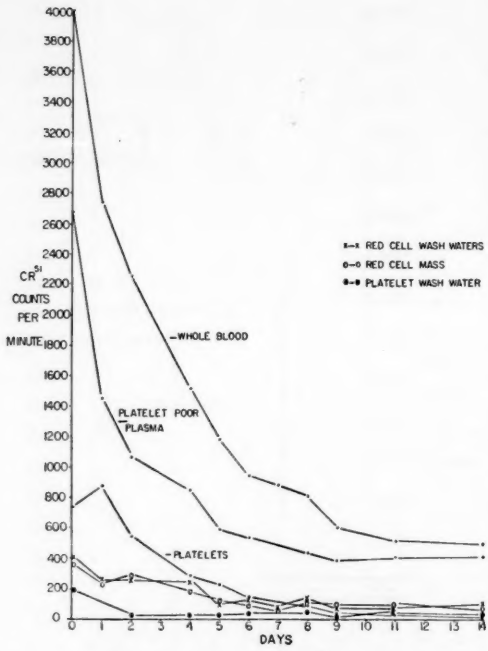

FIG. 7. PLATELET SURVIVAL OF $Cr^{51}Cl_3$—FEMALE, B. G.

$Cr^{51}Cl_3$ labeled platelet concentrates may be followed for nine days in this study. This chromium salt offers no advantage over $Na_2Cr^{51}O_4$ and prolongs circulating radioactivity by the associated binding of plasma proteins.

recipient received $Cr^{51}Cl_3$ labeled platelets, and the results are plotted in Figure 7, showing that the radioactivity of the Cr^{51} platelet button could be followed for nine days. However, the high residual plasma activity emphasizes the value of $Na_2Cr^{51}O_4$ for *in vitro* labeling.

The selective separation of platelets by centrifugation was demonstrated in one recipient who inadvertently received a test dose of KI^{131} for evaluation of thyroid function. Despite the marked increment of radioactivity in the whole blood on the sixth day of study, a linear survival curve of Cr^{51} platelet activity could be followed for 10 days (Figure 8).

Specificity of platelet labeling

Observation of the plotted curves of Cr^{51} activity in the recipients indicates that there is no binding of the recipient's platelets. Measurable radioactivity of the platelet button disappears between 9 to 11 days, and no residual activity will be found in the separated platelets thereafter. Platelet-poor plasma, separated red cells, and the white cell buffy coat suspended in platelet-poor plasma, were labeled with 30 microcuries of $Na_2Cr^{51}O_4$, and after incubation ascorbic acid was added to each preparation. These suspensions were then transfused into normal recipients. Blood samples from the recipients showed no radioactivity in the separated platelet buttons.

Urinary excretion of Cr^{51}

The Cr^{51} urinary excretion was measured during the studies of $Na_2Cr^{51}O_4$ labeled platelets in four normal recipients (Table II). The urinary radioactivity is expressed as the percentage of total counts of Cr^{51} in the infused platelet concentrate. A large portion of the Cr^{51} activity infused into the recipient is excreted during the first 24 hours after the platelet transfusion. The variability in the initial 24 hour excretion reflects the efficiency of platelet labeling and is a measure of the residual Cr^{51} in the surrounding plasma that was not removed completely after labeling. The excretion was followed for 22 days in each instance, and the percentage of Cr^{51} left within the body ranged from 19 to 35 per cent of the injected dose. In Table III the absolute values of Cr^{51} derived from the 300 microcurie labeling dose are tabulated on the basis of the per cent of platelets labeled. After 22 days, 6 to 18 microcuries of Cr^{51} activity remained. Assuming a continued daily excretion of 0.5 per cent of the original Cr^{51} activity, the infused Cr^{51} will be excreted in 80 to 90 days.

TABLE II

Radioactivity excreted in the urine and remaining in the body expressed as per cent of the radioactivity infused in the platelet concentrate

Recipient	Excreted first 24 hours	Excreted first 22 days	Remaining after 22 days
	%	%	%
W. F.	16.8	38.6	34.5
T. H.	45.0	65.0	19.7
D. N.	37.6	61.0	22.0
J. B.	24.0	48.0	29.0

DISCUSSION

The life span of Cr^{51} labeled human blood platelets is 9 to 11 days according to these studies. The manipulation of blood in plastic bag containers has continued to offer the most satisfactory method for preparing sterile platelet concentrates. The elimination of air phase exposure in the separation procedures assures rapid separation of plasma components after centrifugation, and with practice the labeled platelet concentrates may be prepared in less than two hours.

Recent studies by Campbell, Small and Dameshek (*22*) have emphasized alterations of the metabolic function of human platelets during storage. Platelets labeled with P^{32} demonstrated leakage of phosphorus into plasma during storage; a lesion similar to changes noted in the red cell during preservation studies. These metabolic changes emphasize the difficulties associated with *in vitro* studies, for the platelet function begins to deteriorate to some extent when the blood sample is obtained. The rapid elution of Cr^{51} activity from the labeled platelets *in vitro* must be evaluated in relation to the time involved in making the observation. Certainly, after the infusion of labeled platelets into the recipient, elution cannot be observed by the present methods.

Throughout the present technique the platelets have been maintained in a plasma medium, since platelets washed in physiologic saline show morphological alterations. Oxidase activity of platelets and the ability to promote clot retraction diminish rapidly if platelets are suspended in saline (*22*). During the present investigation one transfusion was done with Cr^{51} labeled platelets suspended in physiological saline instead of plasma. Although a normal life span of platelets could be followed in the recipient's circulation, the low radioactivity of the platelet button (15 per cent of the whole blood activity) suggested that the majority of platelets had suffered irreversible damage in the saline solution.

The low Cr^{51} activity of the separated platelets in the recipient during the first few hours after infusion has not been explained completely. A review of the platelet counts of thrombocytopenic

TABLE III

The radioactivity infused in the platelet concentrate and left in the body

Recipient	Radioactivity of infused platelet concentrate expressed as per cent of the initial tagging dose (300 μc.)	Radioactivity infused	Radioactivity remaining in body 22 days after infusion
	%	μc.	μc.
W. F.	16	48	17
T. H.	22	66	13
D. N.	9	27	6
J. B.	21	63	18

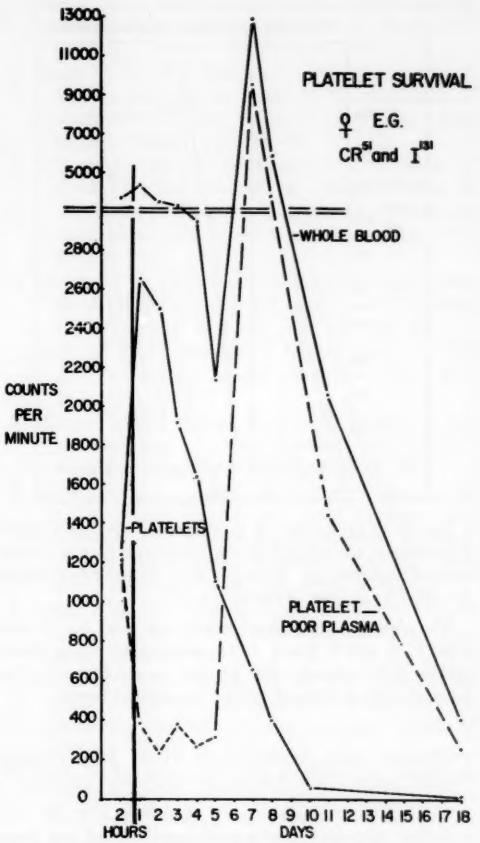

FIG. 8. FAILURE OF KI^{131} TO AFFECT PLATELET SURVIVAL CURVE

On the sixth day of this study, the recipient received KI^{131} orally to evaluate thyroid function. The separated platelet button radioactivity was not influenced by the circulating I^{131}. Although the measurements are plotted as Cr^{51} activity, the whole blood and plasma reflect the presence of Cr^{51} and I^{131}.

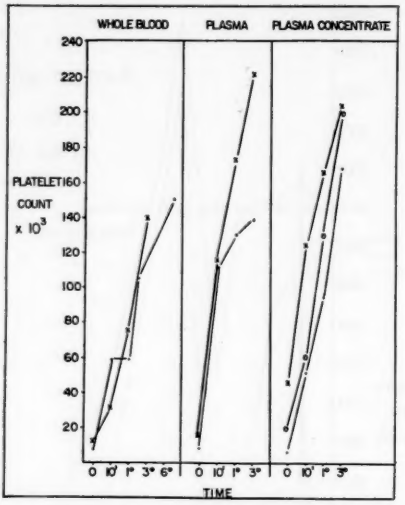

FIG. 9. TABULATION OF PLATELET COUNTS IN THROMBOCYTOPENIC RECIPIENTS AFTER RECEIVING DONOR PLATELETS COLLECTED IN PLASTIC BAG EQUIPMENT WITH Na_2EDTA AS THE ANTICOAGULANT

The platelet counts after infusion are listed for 10 minutes, 1, 3 and 6 hours. The infusion of whole blood, platelet-rich plasma, and platelet concentrates is associated with a delayed mixing for several hours.

recipients after transfusions from polycythemic donors with thrombocytosis has demonstrated a similar phenomenon. The gradual rise in circulating platelets in the peripheral blood has been plotted in Figure 9. In each instance Na_2EDTA was used as an anticoagulant. Despite the rapid collection of whole blood in plastic bags and reinfusion without manipulation, a delayed appearance of circulating platelets was noted. These changes were not noted if whole blood was collected and infused in small aliquots with silicone-coated syringes. The mere collection of platelets in an anticoagulant appears to alter the membrane. In addition, centrifugation of the platelets for concentration and labeling may further effect platelet viability. In thrombocytopenic recipients only about 50 per cent of the theoretical platelet yield has been recovered. In the present study there has been no opportunity to compare directly the Cr^{51} platelet activity with platelet yield, for such a study must be done in a recipient with amegakaryocytic thrombocytopenia.

Preliminary investigations by body surface-counting with a scintillation probe have suggested transient increased radioactivity over the lungs and liver areas. Possibly alterations in the platelet membrane during the labeling procedure produce a sticky surface with transient sequestration in the reticulo-endothelial tissues. With nutritional and environmental equilibration, the platelet regains normal function and is released into the circulation. Separate populations of labeled platelets do not appear as may be noted in the curves of peripheral Cr^{51} platelet activity. The "peaking" of platelet activity during the first 24 hours has been observed in all of the transfusion studies. Preliminary observations suggest that this 24 hour peak disappears in the presence of thrombolytic plasma factors as noted in idiopathic thrombocytopenic purpura and in platelet immunization from multiple transfusion (2).

A review of the ABO blood groups in these studies indicates that incompatible transfusions also had 9 to 11 day survival periods. Although the blood platelets appear to have grouping parallel to the red cell ABO classification (23), individual platelet transfusion studies are not affected by the incompatibilities. There is inadequate evidence to demonstrate that shortened platelet survival periods are related to ABO blood group sensitization.

The measurement of labeled platelet life span in the early transfusions of this study suggested an exponential curve when the values were plotted. Random destruction was assumed to exist following metabolic alteration of the donor platelets during the labeling procedure. With improved proficiency in the preparation of the platelet concentrates, the labeled platelet activity appears to be linear, to suggest that the platelets have disappeared by senescence. This linear loss of platelet radioactivity during the first nine days is helpful evidence to exclude any pathological effect of metallic chromium. During the last 48 hours that the labeled platelets may be followed in the peripheral circulation, the decline in Cr^{51} activity levels off. There is no adequate explanation for this observation, although this phenomenon may suggest changes in the binding of the Cr^{51} to the platelet membrane and consequent elution. The full analysis of these changes may be evaluated by simultaneous study of platelets labeled with

$Na_2Cr^{51}O_4$ and DFP^{32}. In such instances the DFP^{32} incorporation *in vivo* would be the life span platelet reference, much as the Ashby differential agglutination survival period has been used to compare $Na_2Cr^{51}O_4$ life span of red cells.

The present procedure with $Na_2Cr^{51}O_4$ offers a reproducible method as observed in 30 normal adult subjects. The ease of preparing labeled platelets will allow more complete interpretation of thrombocytopenic states. At the present time the method offers no therapeutic advantage over direct transfusion of platelet-rich whole blood or plasma concentrates. However, labeled platelets may be prepared with ease from the average donor without seeking polycythemic donors with thrombocytosis. The life span of platelets measured with DFP^{32} has the most reasonable physiologic application for the normal recipient, but large volumes of venous blood are required to separate an adequate number of platelets. The amount of P^{32} activity in the labeled platelets of thrombocytopenic subjects would not allow reproducible determinations.

SUMMARY

A standardized technique has been developed to evaluate the life span of transfused blood platelets in normal recipients. *In vitro* measurements indicate that radioactive sodium chromate will bind to human blood platelets suspended in plasma. The factors related to time, temperature and concentration of sodium chromate for labeling platelets *in vitro* have been presented. $Na_2Cr^{51}O_4$ labeled platelets have been infused into 30 normal recipients. Determination of the radioactivity in the recipient's platelets separated by differential centrifugation indicates that the transfused platelets have a survival period of 9 to 11 days. No relabeling of the recipient's platelets or other blood elements was observed. The platelet radioactivity in the recipient declines for the most part in a linear fashion to suggest that this method does measure the life span of the transfused platelet.

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MANGANESE METABOLISM IN MAN: RAPID EXCHANGE OF Mn^{56} WITH TISSUE AS DEMONSTRATED BY BLOOD CLEARANCE AND LIVER UPTAKE [1]

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Despite the well-known physiological importance of manganese (1–7) and its implication in some disease states (2, 8–10), the exact nature of its metabolic role remains undefined (11, 12). In mammals manganese concentrates in organs rich in mitochondria, notably the liver (2–7, 11, 13, 14); and previous work from this group (11) has shown these organelles to be the major pool of radiomanganese turnover shortly after injection into animals. *In vitro* traces of the element have widespread biochemical activity (2, 3, 15–19), especially in relationship to mitochondria (20) and those of their enzyme systems that carry out oxidative phosphorylation (16, 21–26).

The present study of blood clearance and of radiation measured at the body surface following Mn^{56} injection in man is an attempt to describe and to determine the kinetics of this turnover, which appears to correlate with mitochondrial function. From the analysis of these data in relation to the work of others it is also possible to gain insight into the mechanisms of transport directly from the blood. Our results further suggest that most of the normal body manganese is readily exchangeable and is not bound in metalloproteins or other highly stable configurations.

EXPERIMENTAL

Radioisotopic tracer. Mn^{56} (half-life: 2.58 hr.) [2] was generated in the Brookhaven research reactor. One ml. aliquots containing 11.1 μg. of $MnSO_4 \cdot H_2O$ (3.6 μg. of Mn^{++}) in saline and representing 15 to 20 microcuries of Mn^{56} [3] were injected into patients. The radiation decay of other aliquots was followed for 48 to 72 hours with a well-type NaI(T1) scintillating crystal and scaling circuit. The resultant activity curves indicated the only contaminating radioelement to be a small amount of Na^{24} (about 0.5 per cent of the Mn^{56} activity at injection time), which was ignored in the subsequent treatment of the data.

Route of administration and withdrawal of blood samples. Following an overnight fast, patients received Mn^{56} tracer intravenously. Thereafter serial venous blood samples of approximately 3 ml. volume were withdrawn from the contralateral arm. Samples were promptly assayed for radioactivity and weighed. Corrections for radioactive decay were then made. Frequent samples were obtained during the first few minutes after injection, but later sampling frequency was decreased gradually to intervals of approximately five minutes.

Surface counting technique. Concurrently with the withdrawal of blood samples, radiation was monitored over the body surface with collimated detectors whose outputs were transcribed automatically.[4] As anticipated from animal data (2, 4–7, 11), preliminary scanning following Mn^{56} injection revealed localization of the greatest radioactivity in the region of the liver. Accordingly, one scanner was positioned on the skin surface over the liver area, usually about 3 cm. above the costal margin in the right midclavicular line ("liver area" counter). A second counter was located over the right femur about 18 cm. above the patella ("thigh" counter). This was taken to be a "reference area" that would not reflect active visceral metabolism or selective manganese uptake.

The exact distribution of Mn^{56} *in situ* was unknown. Thus absolute calibration of the body surface counters with laboratory standards was impossible. Furthermore, two scanners of different sensitivity were used. Empirically, the response of one specific counter in a fixed position and orientation was taken as standard. The two scanners then were intercalibrated at late times, when the levels of external radioactivity had reached virtual equilibrium.

Standardized units of radioactivity. Intercomparison

[1] This research was supported by the United States Atomic Energy Commission.

[2] The radioemission spectrum of Mn^{56} is complex, but the decay scheme includes several energetic gamma rays (most prominent at 845 Kev) (27) of such penetrating power that correction for self-absorption of radiation emanating from within the body was unnecessary. Bremsstrahlung associated with abundant high-energy negatrons provides a further source of externally detectable radiations.

[3] This presented to the recipient a total-body radiation dose of less than 4 m-rad (28).

[4] Voltage signals from conventional scaling circuits were recorded on separate channels of a standard polygraph, and the resulting frequency record was integrated manually and plotted as an amplitude curve (*cf.* Figure 1).

TABLE I

Patient data

Experiment	Date	Patient	Unit no.	Sex	Age	Major diagnosis
1	11/21/55	F. McD.	7090R	M	60	Cancer with metastases (some to liver)
2	11/22/55	(F. McD.)*				
3	12/19/55	R. E.	7107R	F	40	Wilm's tumor with metastases (some to liver)
4	3/30/56	F. M.	7275R	M	42	Cancer with metastases (none apparent in liver)
5	5/24/56	M. M.	7376R	M	65	Paralysis agitans—? idiopathic
6	6/8/56	A. B.	7343R	F	60	Paralysis agitans—postencephalitic; diabetes
7	7/26/56	J. McK.	7284R	F	42	Paralysis agitans—? postencephalitic
8	8/23/56	(A. B.)				
9	9/11/56	F. N.	7785R	F	51	Paralysis agitans—? idiopathic; diabetes
10	9/20/56	B. S.	7826R	F	71	Hypertensive vascular disease—recent cerebral vascular accident
11	9/21/56	L. M.	7656R	M	67	Polycythemia vera—old cerebral vascular accident
12	10/2/56	R. B.	7159R	M	48	Essential hypertension
13	10/9/56	G. M.	7835R	M	48	Rheumatoid arthritis
14	11/30/56	T. D.	7853R	M	60	Diabetes—peripheral vascular insufficiency
15	1/25/57	J. B.	8062R	F	47	Paralysis agitans—? postencephalitic
16	2/1/57	(T. D.)				
17	2/15/57	(B. S.)				
18	2/28/57	H. C.	8103R	F	57	Paralysis agitans—? idiopathic
19	3/5/57	(H. C.)				

* ( ) indicates a second entry of a patient previously cited.

of data was facilitated by the reduction of all radioactivity values to standardized units (*29*, *30*). Experimental readings were adjusted for ambient background and then were corrected for radioisotopic decay, variations in tracer dosage and difference in body weight. Thus blood radioactivity is in units of counts per minute per gram of whole blood per microcurie of administered isotope per 70 kilograms of body weight, while external radiation (radioactivity) is in different units of counts per minute per microcurie per 70 kilograms. The empirical ratio relating the two different units remained fixed within the range of experimental reproducibility, so relative changes in the time courses of blood and body surface data could be compared, as in Figure 1.

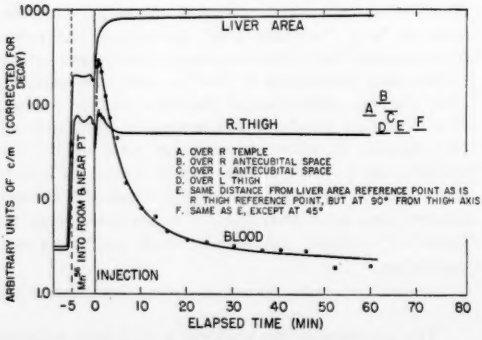
1000
100
10
1.0
ARBITRARY UNITS OF c/m (CORRECTED FOR DECAY)
LIVER AREA
R. THIGH
A B C D E F
A. OVER R TEMPLE
B. OVER R ANTECUBITAL SPACE
C. OVER L ANTECUBITAL SPACE
D. OVER L THIGH
E. SAME DISTANCE FROM LIVER AREA REFERENCE POINT AS IS R THIGH REFERENCE POINT, BUT AT 90° FROM THIGH AXIS
F. SAME AS E, EXCEPT AT 45°
BLOOD
Mn⁵⁶ INTO ROOM & NEAR PT
INJECTION
-5 0 10 20 30 40 50 60 70 80
ELAPSED TIME (MIN)

FIG. 1. REPRESENTATIVE EXPERIMENTAL DATA: PATIENT J. B.

Since the injection site is closer to one counter than the other, note the different readings when Mn^{56} is in position for infusion.

Radiation scatter. External radioactivity over the liver usually reached values 10 to 20 times greater than those over the thigh (*cf.* Figure 1), indicating that radiations scattered from Mn^{56} outside the field of view of this counter were relatively insignificant. Therefore, analyses of liver area data neglected scatter.

RESULTS

Blood and liver area measurements were performed on 18 occasions in 14 individuals (Table I). In one additional instance (Experiment 17) only blood data were obtained.

A. The disappearance of injected Mn^{56} from peripheral venous blood

Compiled blood disappearance data are presented in the scattergram of Figure 2. Beyond 22.5 minutes along the abscissa the regression represented by the data is regarded as diminishing exponentially. A straight line has been fitted to the points in this interval by the method of least squares, using logarithms of the blood radioactivity values.[5] Data from times less than one

[5] The resultant least squares regression line of the logarithms (base 10) of the radioactivity against time has a zero-intercept of 0.6375 (log of 4.340), a slope of 0.01007 (log of exp-0.0232), a standard error of estimate

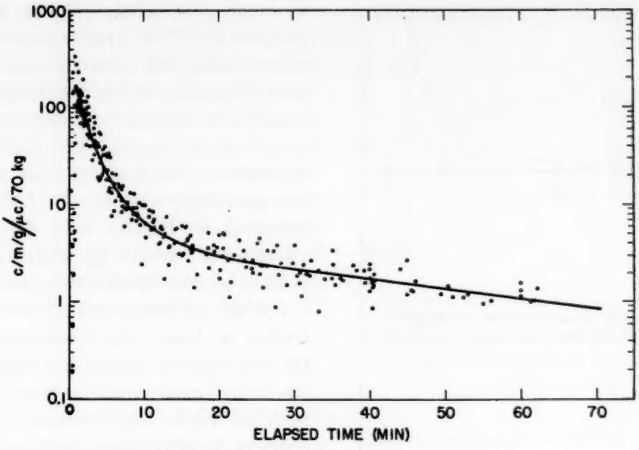

FIG. 2. CLEARANCE OF MN^{56} FROM THE BLOOD

minute are excluded from the calculated curve, because in individual experiments the peak of radioactivity in the peripheral blood occurs at about one minute or less, with the precise time depending on factors pertinent only to the circulatory mixing process. The remaining blood data between 1 and 22.5 minutes of time have been approximated by a best eye-fit curve, which is drawn to fit smoothly with the least squares regression line at later times. The resultant curve (Figure 2) is termed the "blood clearance curve," whose ordinate values are in standardized blood radioactivity units, as defined earlier. These units have dimensions equivalent to the fraction of administered radioactivity remaining in a gram of blood, after adjustment for variations in body weight.

B. The appearance of Mn^{56} radiation over the body surface

Data from a representative experiment are presented in Figure 1.

1. Thigh area radioactivity. The characteristic record of radiation over the thigh reveals an early rise and decay followed by a plateau of activity essentially unchanged for the remainder of the observation period.[6]

2. Liver area radioactivity. The radioactivity in the liver region shows a rapid early rise to a high value which increases only very slightly thereafter. A composite representation of the liver data has been compiled (Table II and Figure 3), using the arithmetic means of the values of individual records at various times after injection.

CALCULATIONS AND DISCUSSION

Analysis of the blood clearance and liver uptake data reported here allows characterization of some important aspects of the metabolic behavior of body manganese.

A. The dynamic metabolic state of manganese

In this portion of the discussion the active turnover of manganese from the systemic circulation will be identified with a rapid transport within the liver and more generally throughout the body.

1. Rapid movement of manganese from the blood. The carrier manganese injected along with the Mn^{56} was less than 3 per cent of the normal plasma or blood content (31). Hence it represented an insignificant perturbation of the endogenous physiology. Therefore, a steep decline in

(σ) of 0.1377 (*i.e.*, plus 37.1 per cent and minus 27.2 per cent about the mean), and a product moment coefficient of correlation (r) of minus 0.632 ($p < 0.001$).

[6] The early rise and fall of thigh radioactivity is compatible with the presence of blood-borne Mn^{56} within the limb. The succeeding plateau appears to reflect radiation scattered largely from the accumulation of tag in the liver and other viscera. This conclusion was based on the virtual lack of change in the "plateau" reading of Figure 1 when the counter was moved in a circle with the liver as center (Curves D, E, and F of Figure 1).

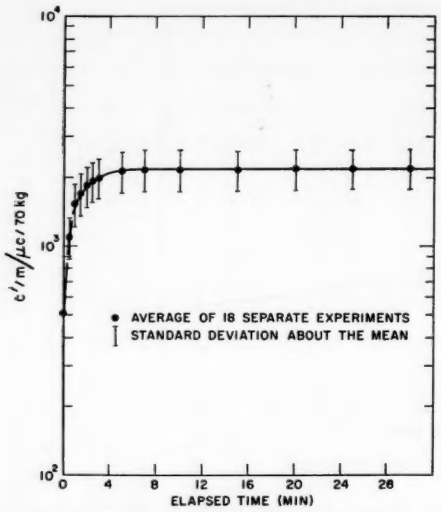

FIG. 3. RADIOACTIVITY OVER THE LIVER AREA AFTER INTRAVENOUS Mn^{56}

Note that the arbitrary counting units of the reference external counter are designated by C′ to distinguish them from the readings, C, of the well-counter utilized in the blood work. The length of the bar represents plus-minus one standard deviation about the mean.

blood radioactivity would reflect the rapid movement of both tracer and endogenous circulating manganese to extravascular sites.

Description and significance of the blood clearance curve. Radioactive tracers generally mix with substantially the entire blood volume well within one minute following intravenous injection (29, 30, 32). Figure 2 indicates that much of the radiomanganese initially in the blood is cleared within a minute of virtual mixing, with very little remaining by 45 minutes. However, manganese is excreted much more slowly than this (12, 33–37): Hence the high dilution of initial blood radioactivity implies rapid concentration of the element at certain sites. From partition studies of endogenous and tracer manganese (11, 14), these sites can be presumed to be intracellular.

The blood clearance curve of Figure 2 has been analyzed into three exponentially decaying components (38). It is well represented analytically by the equation

$$a(t) = 265e^{-0.77t} + 44.1e^{-0.26t} + 4.34e^{-0.023t}, \quad 1)$$

where a(t) is radioactivity in standardized units (Figure 4). The first exponential component is comparable to the aperiodic time relation of Sheppard, Overman, Wilde and Sangren describing the rapid blood clearance of indicators (30). The last component is identical with the exponential (antilogarithmic) form of the least squares regression line previously calculated. Although the experimental curve has not been determined for $t < 1$ minute, the "virtual" blood clearance can be extrapolated to zero (injection) time.

Rapidity of transcapillary exchange. The steep decline of blood radiomanganese represented by the first term of Equation *1* implies that in reality the tracer never quite achieves uniform mixing with the entire blood volume, even after several minutes, because along each capillary there must be a strong gradient of radioactivity (29). Furthermore, should this gradient reach zero before the entire length of the capillary be traversed, the transendothelial passage of manganese would actually be more rapid than the calculated value represented by the exponential coefficient, 0.77-minute^{-1}. Keeping in mind the distinction between the net movement of small molecules produced by large concentration gradients and other influences and the rates of transfer *in both directions* through a membrane (39), it is likely that the transcapil-

TABLE II

External radioactivity of the liver (18 experiments)

Elapsed time *min.*	Mean value *C′*/min./μc./70 Kg.*	Standard deviation *C′/min./μc./70 Kg.*
Injection time	507	152
0.5	1,096	223
1.0	1,528	323
1.5	1,698	352
2.0	1,830	362
2.5	1,916	374
3.0	1,978	383
5.0	2,104	418
7.0	2,138	427
10	2,143	427
15	2,132	411
20	2,157	419
25	2,163	415
30	2,171	410
40	2,188	400
50	2,213	373
60	2,216	265

* The units C′ refer to counts recorded by the external radiation monitor. These units are to be distinguished from the units, C, which refer to counts recorded by the counter used to determine blood radioactivity.

lary movement is in reality exceedingly rapid, because for water and certain other small molecules the transfer in both directions through the vessel wall occurs at rates that are prodigious in comparison with their rates of *net* transport from the vascular space (39).

The overall turnover rate of blood manganese, therefore, has also been estimated for comparison with that of other small ions which have been studied in detail. R is defined as the fraction of the total intrinsic blood manganese that leaves the vascular compartment in one minute, where

$$\frac{[d\,a(t)]}{(dt)_0} \Big/ a_0 = R = (-\lambda_1 a_1 - \lambda_2 a_2 - \cdots - \lambda_n a_n)/a_0, \quad 2)$$

with a_0 equal to the sum of a_i (29, 32). After substitution from Equation *1*, R becomes about 69 per cent.

This value of R calculated for manganese is roughly the same as for other electrolytes, except potassium, as measured in various species (29). Since diffusion or simple filtration, acting alone, would pass all electrolytes at nearly the same rates (29), this similarity is consistent with a diffusion mechanism of transcapillary transport. By contrast, potassium is exchanged much more rapidly than manganese and other ions (R = 225 per cent in the rabbit), and for this case more complex transfer mechanisms have had to be invoked (29, 40).

2. Important role of the liver. Prompt hepatic uptake and retention of injected radiomanganese demonstrate that the liver partakes importantly in the swift exchange of circulating manganese with intracellular sites; however, significant extrahepatic influx of radiomanganese occurs also. This conforms with the organ distribution of Mn^{56} in animals (11, 36, 41).

Intraportal injection studies with tracer manganese in animals demonstrate nearly complete removal of tag in a single transhepatic passage (42). In such a case the fraction of tracer disappearing from blood per minute approaches f/T, where f is the fraction of the entire systemic arterial outflow traversing the liver and T is the time for a single blood volume of tracer to circulate through the heart and appear in the arterial supply or the portal inflow of the liver. Then when other symbols are employed as before,

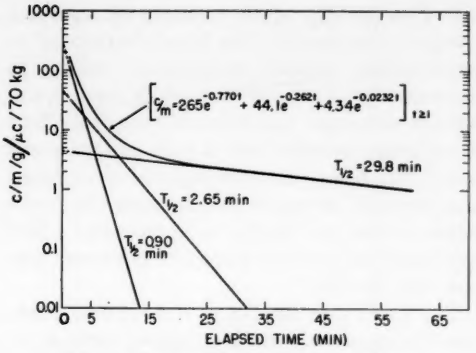

FIG. 4. COMPONENT ANALYSIS OF BLOOD CLEARANCE OF Mn^{56}

$$a(t)/a_0 = e^{-(f/T)t} = e^{-\lambda_1 t}, \quad 3)$$

since f/T is equal to λ (43).

In man the resting cardiac output has been reported variously as about 3 to 6 liters per minute (44, 45), with a total blood volume of 5.0 to 5.5 liters (44, 46). Therefore, one minute represents an apparent average circulation time, T.[7] λ_1 can be taken from Equation *1*, and f then becomes $\lambda_1 T$, or 0.77. However, it is apparent that 77 per cent of the cardiac output cannot traverse the liver, whose total blood flow has been calculated by several methods as about 1,100 to 1,800 ml. per minute (48, 49). It is more reasonable to assume that Mn^{56} is removed from the blood by capillary beds elsewhere equally as fast as it is cleared by the liver.[8]

[7] The exact determination of T is impossible, since its physical meaning is obscure. Different circulatory pathways may not possess identical circulation times, so several circuits of blood over some shorter routes may occur while a single circuit is being made through some longer pathway (29, 47). For example, the shortest complete circulation time in man is about 21 seconds (range of 12 to 26 seconds) (44); whereas the apparent mean circulation time approximates one minute.

[8] In fact, assuming the turnover value of manganese to be about 70 per cent (Equation *2*) with an hepatic blood flow of 1.5 liters per minute (44, 49) out of a total cardiac output of 4 liters per minute (44, 45), it is found that 0.70(1.5/4.0), or about 25 per cent of injected Mn^{56} should accumulate in the liver, provided the intrinsic hepatic clearance is efficient. As a check upon the present calculations, this figure agrees reasonably with the distribution found by Maynard and Cotzias (11, 42) and with that estimated from the data of others (34, 41).

3. Homogeneity of all pathways of rapid exchange of manganese. The blood clearance of an intravenously injected radioisotope reflects its movement to *all* organs with which there is significant exchange. An identical time-dependence of radiation recorded over a body surface would imply that these same mechanisms of exchange also produced isotope retention within the tissues whose uptake was thereby being measured. This hypothesis has received general experimental support (48, 50–52).

Blood and liver kinetics of Mn^{56} can be correlated by expressing the liver uptake curve as an enantiogram of the difference between liver activity at a given moment and the final equilibrium ("plateau") value, plotted against time (48) (Figure 5). Then over the first four minutes or so Figure 6 shows the blood and liver kinetics to be virtually identical, and even at later times the differences are small. The clearance of the bulk of injected Mn^{56} from the peripheral blood therefore reflects the same means of manganese transport that account for most of its accumulation by the liver.

Extension of the above reasoning implies that most of the influx of manganese into other organs also proceeds in the same fashion as in the liver. Hepatic uptake has been shown to account for the disappearance of well under half of the Mn^{56} from blood. The parallel activity curves can be explained alternatively only by the existence of extrahepatic pathways of rapid manganese interchange which are significantly different in nature from those existing in liver but which possess, nevertheless, virtually identical overall kinetic behavior. This is unlikely.

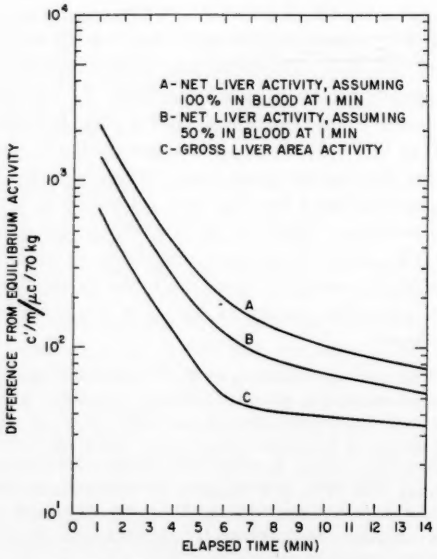
A- NET LIVER ACTIVITY, ASSUMING 100% IN BLOOD AT 1 MIN
B- NET LIVER ACTIVITY, ASSUMING 50% IN BLOOD AT 1 MIN
C- GROSS LIVER AREA ACTIVITY
DIFFERENCE FROM EQUILIBRIUM ACTIVITY c'/m/μc/70 kg
ELAPSED TIME (MIN)

FIG. 5. APPROACH OF LIVER ACTIVITY TOWARD EQUILIBRIUM

The liver area counter also detects Mn^{56} in tissue adjacent to the liver. At the time of maximum blood radioactivity (*ca.* one min.) Curve A assumes all detected activity to derive from tracer still circulating, while B represents a less severe assumption. A and B are calculated from Figure 3, taking circulating Mn^{56} to decline as in Figure 2. Intrinsic hepatic radiomanganese uptake presumably lies within the range A-C.

B. The active role of mitochondria in manganese metabolism

In animals early radiomanganese distribution is characterized by preferential mitochondrial accumulation (11, 42). In the present experiments a single mode of manganese metabolism has been

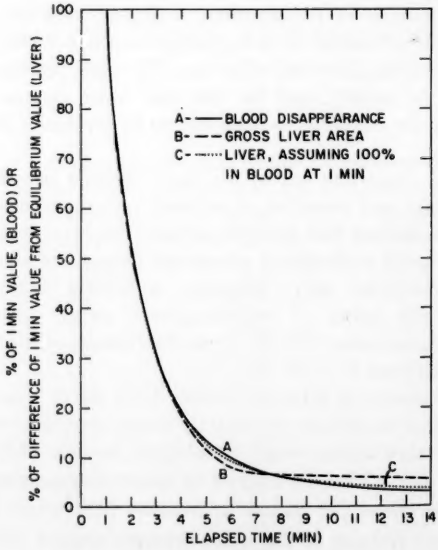
A- —— BLOOD DISAPPEARANCE
B- — — GROSS LIVER AREA
C- ····· LIVER, ASSUMING 100% IN BLOOD AT 1 MIN
% OF 1 MIN VALUE (BLOOD) OR
% OF DIFFERENCE OF 1 MIN VALUE FROM EQUILIBRIUM VALUE (LIVER)
ELAPSED TIME (MIN)

FIG. 6. COMPARISON OF EARLY BLOOD AND LIVER KINETICS

The kinetics of Mn^{56} movement in blood and liver are nearly congruent regardless of whether liver area (B) or calculated organ uptake data (C) are employed.

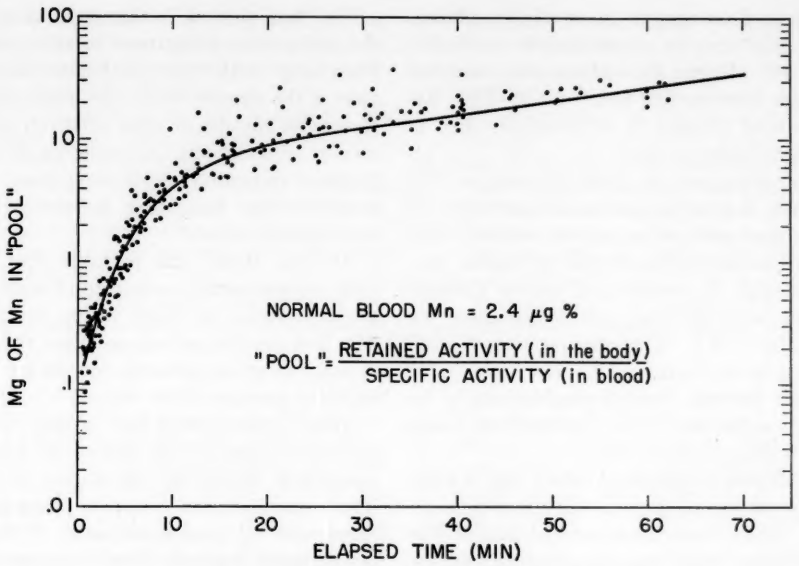

FIG. 7. "APPARENT" EXCHANGEABLE MN "POOL"

shown to determine overall tracer distribution in the body shortly after injection. It therefore appears that in man the mitochondria of all organs participate importantly in the rapid interchange of endogenous tissue manganese with that circulating in the blood.

It has been shown earlier that the first of the analyzed exponential components of the Mn^{56} blood disappearance curve probably represents transcapillary exchange, and later it was inferred that mitochondrial assimilation dominates the transfer of tracer during the first hour. Since the first term of the blood curve has been otherwise identified, whereas the third term does not suggest an exchange as rapid as the rate of mitochondrial uptake, it is probably the second component of the clearance curve (*cf.* Figure 4 and Equation *1*) that reflects strongly the movement of injected radiomanganese into the mitochondria themselves. However, that component's slope and intercept cannot as yet be interpreted in a quantitative fashion.

Insofar as rapid manganese exchange in tissue signifies mitochondrial function, some measure of this intracellular parameter may be determined in man, using the methods reported here. The total mass or activity of mitochondria [9] may be indicated by the initial portion of the radiomanganese blood clearance curve, and particularly by the second exponential component analyzed from it; and the liver area uptake may provide a similar measure of hepatic mitochondria. Independent substantiation would be highly desirable, but present cellular fractionation techniques (11) lack sufficient time resolution to confirm this relationship directly in animals.

C. The labile physico-chemical status of body manganese

Each of the recognized essential mineral micronutrients other than manganese [10] has been demonstrated as a strongly bound intrinsic component of one or many naturally occurring metalorganic compounds (2, 16, 18, 19, 53). In contrast, no manganese metalorganic component has yet been definitely identified in animal tissues (2, 16, 18, 19, 53).[11]

[9] Exclusive of brain, because of the intervening blood-brain barrier.

[10] Iron, cobalt, copper, zinc, and molybdenum.

[11] Since this paper was submitted for publication, evidence has been educed that strongly suggests the presence

The mass of manganese apparently interchanging with Mn^{56} can be represented by a dilution "pool" (54) (Figure 7). Manganese excretion is negligible over the first hour (12, 33–37). Retained activity (Figure 7) is therefore taken as the entire radioisotope dose.

Data on manganese in blood are meager (3); but recently Bowen has perfected a technique of improved resolution and sensitivity wherein small blood samples are radioactivated by reactor neutrons, followed by conventional carrier chemical separation processes and then by counting the isolated Mn^{56} (31). The concentration of manganese has been determined thereby to be 2.4 ± 0.8 μg. per 100 ml., divided roughly equally between cells and serum. This represents the lowest reported value (3, 4, 55, 56).

In the absence of individual values, that of 0.024 μg. per gram from Bowen (31) has been assumed. Thence from the individual data of Figure 2 a dilution "pool" was calculated (Figure 7).

Recent reports cite a total body manganese content of 11 to 20 mg. (2, 46). Extrapolation from human (7) and animal (13) tissue measurements places the body manganese at 9.5 to 12.0 mg. and 22.5 to 27.2 mg., respectively. In any case, by 45 to 60 minutes the Mn^{56} interchanges with a pool representing roughly all the endogenous body manganese (Figure 7).[12]

The rapid dilution of tracer (Figure 7) indicates the absence of a significant nonexchangeable portion of the total body manganese, and conversely, that most body manganese must reside in relatively labile metal-protein complexes or similar configurations compatible with its known chemistry (13–17).

SUMMARY AND CONCLUSIONS

Combined studies of blood clearance and radiation measured over the liver in humans following the intravenous injection of tracer doses of Mn^{56} showed a consistent pattern. The transfer of manganese from blood into intracellular sites was rapid throughout the body.

The data showed further that most or all of the endogenous manganese became available for interchange with tracer within an hour. Hence most of the element within the body must exist in dissociable chelates or other relatively labile intracellular combinations. Considering the constancy of tissue concentrations of manganese, this demonstrates that manganese metabolism is under homeostatic control.

At least three, and probably four, apparent body compartments participate to some extent in the distribution of Mn^{56} within the first hour. The first component analyzed from the blood disappearance curve probably reflects the net transcapillary passage of the element.

Tracer concentration falls steeply in the capillaries, and about 70 per cent of the normal blood manganese leaves the circulation each minute. The transendothelial transport of manganese appears to be by passive diffusion. It follows that net turnover from the blood is perfusion-limited, despite subsequent intracellular concentration.

Identical mechanisms appear responsible for most of the swift inflow of manganese into liver and simultaneously into other organs. Mitochondrial uptake throughout the body is regarded as underlying the bulk of this rapid tissue retention, which largely determines a second component analyzed from the blood data as well.

A mitochondrial role is in accord with the known biochemistry of manganese *in vitro*. Therefore, the procedures reported here are proposed as a method for measuring mitochondrial mass or function in patients.

ACKNOWLEDGMENTS

The authors wish to acknowledge the expert assistance in this work of J. J. Greenough and H. Hamel.

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THE RELATIONSHIP BETWEEN AIRWAY RESISTANCE, AIRWAY CONDUCTANCE AND LUNG VOLUME IN SUBJECTS OF DIFFERENT AGE AND BODY SIZE[1, 2]

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The resistance of the airways to the airflow through them depends on the radius, length and number of the airways. It has long been apparent (1, 2) that the bronchi are distended as well as lengthened in the inspiratory position of the lung. These two changes in the dimensions of these airways would have opposite effects on their resistance to airflow, lengthening increasing resistance and widening reducing it. More recent measurements of airway resistance have shown that it is reduced in the inspiratory position of the lung (3, 4, 5) and is more in individuals with small lungs (6).

The body plethysmograph affords a good technique for comparing changes in airway resistance with changes in lung volume in an individual, since each measurement of airway resistance includes as an essential stage in its derivation, a measurement of intrathoracic gas volume. It therefore seemed desirable to explore further the relationship between airway resistance and lung volume at different degrees of inflation of the lung in normal subjects.

METHODS

Airway resistance was measured by the method previously described by DuBois, Botelho and Comroe (7). The method has the advantage that the pressure changes in the body plethysmograph reflect changes in alveolar pressure rather than changes in pleural or esophageal pressure. The resulting measurements are therefore assessments of pure airway resistance uncomplicated by the effects of tissue viscosity which are included with airway resistance when it is measured by means of changes in esophageal pressure.

The method previously described has been somewhat simplified by eliminating photographic recording of the oscilloscope. The oscilloscope face is now filled by a plexiglass disc on which are ruled parallel lines. While the trace is being made on the oscilloscope face the observer rotates the plexiglass disc until one of the parallel lines is aligned with the tracing.[5] The slope of the lines on the disc is read off scales on the oscilloscope face housing. These scales are in terms of *1.* lung volume × airway resistance, and *2.* lung volume. The former scale applies when the subject is breathing freely through the flowmeter and the ordinates and abscissae on the oscilloscope face are, respectively, plethysmograph pressure and airflow. The latter applies when the subject is panting against a closed shutter and the ordinates and abscissae are, respectively, plethysmograph pressure and mouth or alveolar pressure (Figure 1).

Each subject sat in the 600 liter body plethysmograph for a few minutes until the air in the box was warm enough and sufficiently saturated with water vapor for spontaneous changes in plethysmograph pressure over a period of 10 seconds to be negligible. During this time the amplification of the record of plethysmograph pressure was adjusted until a displacement of 30 cc. to or from the plethysmograph gave a deflection of two inches on the oscilloscope face. The subject then put his mouth over the flowmeter shutter assembly which was suspended in the plethysmograph and panted for about six seconds. The observer aligned the plexiglass disc with the midslope of the tracing and read the product (lung volume × airway resistance) off the calibrated scale. The observer then operated a switch, which closed the solenoid shutter at the mouth, and replaced the record of flow rate by one of mouth pressure, on the Y axis of the

[1] This investigation was supported in part under a contract between the Army Chemical Center and the Graduate School of Medicine, University of Pennsylvania.

[2] Presented in preliminary form at the Fall Meetings of the American Physiological Society, 1956.

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[5] A more detailed description of this modification is being prepared. Meanwhile, the diagram (Figure 1) shows the arrangement of apparatus used in the present study. The oscilloscope tracing was sometimes alinear, showing a tendency to curve at its extremities, due presumably to turbulent flow at higher flow rates. The slope which was measured was that of the central part of the tracing, where flow was small (less than 0.5 liters per second), and apparently—from the linearity of the tracing—streamlined. It is for this reason that it is justifiable to use Equation 4 in the discussion (a modification of Poiseuille's equation for laminar flow) in assessing the relative distensibility of the airways and of the lung.

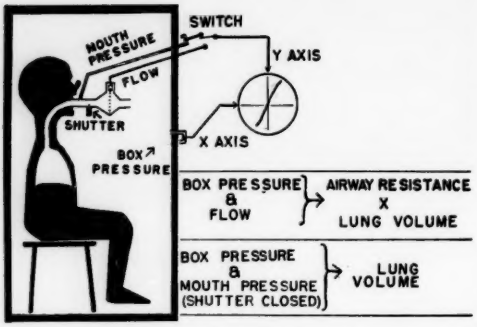

FIG. 1. BODY PLETHYSMOGRAPH AS USED IN PRESENT STUDY, SHOWING THE SITES AT WHICH MOUTH PRESSURE, BOX PRESSURE AND FLOW ARE MEASURED

The two pairs of measurements shown at the right give the numerator and denominator of airway resistance. The switch which connects the Y axis of the oscilloscope to mouth pressure also closes the shutter. The three amplifiers used are omitted.

oscilloscope face. The subject continued his panting efforts against the shutter for about two seconds and then withdrew the mouthpiece from his mouth. In the meantime the observer aligned the plexiglass disc with the fluorescent trace of mouth pressure versus box pressure and read lung volume off the scale.

This procedure was repeated up to 45 times during any one session of a subject inside the plethysmograph. During some observations subjects were instructed to hold their chests in an expiratory position, close to their residual volume. In other cases each subject was instructed to hold his chest in an inspiratory position. In this way measurements of airway resistance were made over a wide range of lung volumes in most subjects. The rapid switch from flow to mouth pressure made it possible to measure the volume of the lung at which the panting was performed and eliminated the chance of a change in the volume between the measurements. Some subjects were studied on more than one occasion.

In the calculation of airway resistance an allowance of 0.78 cm. H_2O per liter per second was made for the measured resistance of the pneumotachygraph used. For reasons explained later airway resistance was expressed in terms of its reciprocal, airway conductance, in units of liters of airflow per second per cm. of water pressure.

It seemed desirable to check on the accuracy of the volume measurements made by the plethysmograph. A bottle was substituted for human lungs. The pressure in the bottle was measured by the mouth pressure manometer. The gas in the bottle was compressed by means of a rubber bulb connected to it, and squeezed by the hand of an operator, sealed in the plethysmograph with the bottle, and holding his breath during observations. The volume of the bottle was measured by the slope of the oscilloscope tracing of plethysmograph pressure against "mouth pressure" by a second operator outside the box, exactly as for human lungs. The bottle contained 0.1 liter of Dreft® detergent solution and 4.0 liters of air. Its volume was underestimated at 3.4 liters by the plethysmograph procedure. But when the Dreft® was partly converted into a fine froth, with bubbles about 1 mm. diameter, and filling the bottle, the gas volume was correctly estimated at 4.0 liters. Evidently the gas in the bottle behaves adiabatically when there is no froth. When intimately mingled with froth the air behaves isothermally during compression, and volumes are correctly estimated by our procedure. The human lung, in its heat exchanging properties, must resemble the froth filled bottle rather than the froth free bottle, and our plethysmograph procedure estimated correctly the volume of the air spaces which exchange heat rapidly with their walls.

RESULTS

All subjects reported here were normal. They were laboratory workers, or the relations of laboratory workers, except for a circus dwarf. One hundred twenty-nine measurements of airway conductance and alveolar volume were made on 8 children studied on 9 occasions, aged between 4

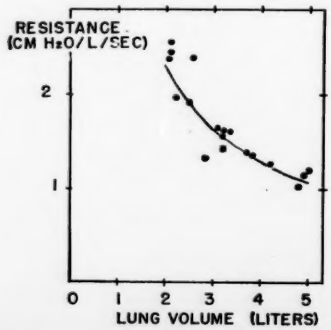

FIG. 2. AIRWAY RESISTANCE PLOTTED AGAINST LUNG VOLUME IN SUBJECT J.Z.

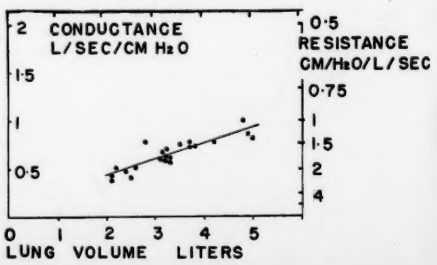

FIG. 3. AIRWAY CONDUCTANCE PLOTTED AGAINST LUNG VOLUME IN SUBJECT J.Z.

and 13 years; 129 measurements were made on 7 women studied on 9 occasions and aged between 16 and 87; 327 measurements were made on 11 men, including one circus dwarf (B), studied on 18 occasions and aged between 23 and 40.

The data for each subject were plotted on a scatter diagram. When airway resistance was plotted against lung volume there was a curvilinear relationship between them (Figure 2). However, when airway conductance was plotted against lung volume there appeared to be a linear relationship.[6] It was decided therefore to relate airway conductance (6) rather than airway resistance to the various lung volumes at which measurements were made in each subject (Figure 3).

The results are given in Table I, which gives the age and physical characteristics of each subject together with the number of observations made at each session on each subject, the mean lung volume, the mean conductance, the regression of conductance on lung volume, the intercept of the regression line on lung volume at zero conductance and the correlation coefficient, at each session on each subject.

The regression lines are plotted in Figures 4, 5 and 6, which express the data on children, women and men, respectively. Each line extends between the maximum and minimum lung volumes at which measurements of airway conductance were made at any particular session on a subject. All lines reflect the same tendency for airway conductance to increase as lung volume increases. It is seen that children show the decreased airway conductance which is compatible with their smaller lung volume. The airway resistance of children is three to five times as much as that of adults.

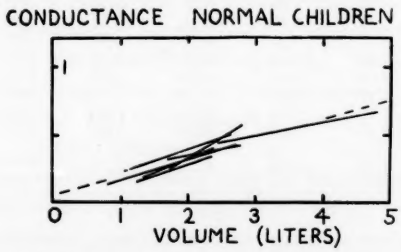

FIG. 4. REGRESSION LINES OF AIRWAY CONDUCTANCE ON LUNG VOLUME IN CHILDREN

The dashed line is the regression line for all data on children.

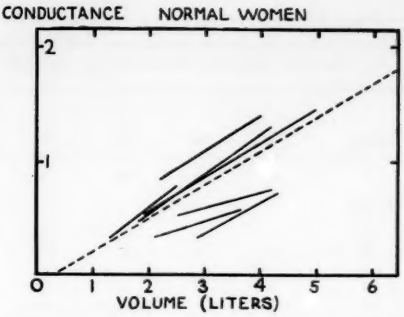

FIG. 5. REGRESSION LINES OF AIRWAY CONDUCTANCE ON LUNG VOLUME IN NORMAL WOMEN

The dashed line is the regression line for all data on women.

Age appears to have little effect on the relationship between airway conductance and lung volume. In Figure 7 the data on five subjects with ages ranging from 4 to 87 are plotted. They all lie close to the same line which expresses the approximate relationship: Conductance = ⅕ lung volume. In children the overall regression of conductance on volume was conductance = 0.14 (volume + 0.49 liter), with a correlation coefficient of 0.83. In women it was conductance = 0.29 (volume − 0.27 liter), with a correlation cofficient

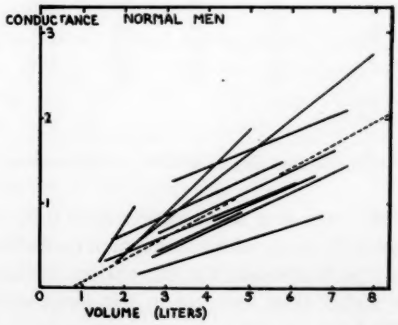

FIG. 6. REGRESSION LINES OF AIRWAY CONDUCTANCE ON LUNG VOLUME IN NORMAL MEN

The dashed line is the regression line for all data on men.

[6] This does not rule out the possibility of curves hidden within the group of points which scatter about the regression line describing the best fit to the general trend. The mid-slope $\Delta C/\Delta V$ sometimes appears less steep than the extremes.

TABLE I

Relationship between airway conductance (C) and lung volume (V) in 26 individuals of different physical characteristics studied at different lung volumes in each individual, some of whom were studied on repeated occasions *

Subject	Age	Sex	Ht.	Wt.	Expts.	Mean V	Mean C	Regression coefficient ΔC/ΔV	Regression intercept V axis	Correlation coefficient
	yr.		*in.*	*lb.*	*no.*	*L.*	*L./sec./cm. H_2O*		*L.*	
Children										
C. G.	9	M	51	68	13	1.76	0.24	0.17	0.06	0.75
D. W.	12	M	61	130	20	1.61	0.26	0.26	0.00	0.90
					15	2.48	0.50	0.32	0.92	0.76
G. G.	9	F	53	72	11	1.69	0.26	0.20	0.39	0.78
J. M.	5	F	47	60	3	0.93	0.17			
A. L.	10	F	60	143	9	2.1	0.36	0.10	−1.48	0.41
C. McN.	4	M	43	45	4	1.2	0.30			
M. C.	13	M	62	135	27	1.87	0.37	0.18	−0.24	0.80
M. S.	14	M	64	140	27	3.76	0.56	0.10	−0.22	0.53
All Children					129	2.24	0.38	0.14	−0.49	0.83
Women										
B. McR.	16	F	64	129	30	3.19	1.15	0.31	−0.48	0.42
E. R.	22	F	62	120	23	3.77	1.09	0.30	0.17	0.56
A. B.	30	F	62	117	12	1.97	0.57	0.38	0.45	0.24
M. H.	23	F	64	110	19	2.74	0.79	0.35	0.51	0.92
B. F.	30	F	67	130	7	3.64	0.55	0.27	1.59	0.73
C. M.	23	F	63	125	13	2.76	0.43	0.17	0.22	0.80
					14	3.04	0.50	0.16	−0.11	0.34
E. M.	87	F	61	100	11	3.29	0.62	0.14	1.16	0.45
All Women					129	3.08	0.81	0.29	0.27	0.54
Men										
A. D.	32	M	73	198	22	4.62	1.38	0.32	0.35	0.85
					22	4.05	1.33	0.39	0.65	0.87
W. B.	37	M	70	160	11	4.65	0.92	0.33	1.87	0.80
					10	4.08	0.75	0.28	1.43	0.90
					15	4.72	0.83	0.21	0.86	0.78
J. Z.	23	M	67	140	22	3.30	0.65	0.17	−0.66	0.89
					9	3.77	0.73	0.24	0.77	0.89
					14	3.59	0.76	0.23	0.22	0.82
					18	3.71	0.82	0.20	−0.50	0.85
R. McN.	34	M	73	168	21	4.94	1.64	0.21	−2.98	0.55
W. B.	36	M	76	202	6	5.36	1.08	0.22	0.40	0.94
R. B.	36	M	67	160	45	5.78	0.47	0.15	0.67	0.87
					19	3.72	0.33	0.16	1.82	0.97
J. R.	35	M	70	170	21	3.54	1.00	0.23	−0.75	0.71
S. K.	40	M	68	170	12	3.63	0.61	0.24	1.02	0.91
E. P.	23	M	73	177	17	3.17	0.99	0.50	1.20	0.92
V. F.	32	M	70	162	36	4.72	1.11	0.24	0.05	0.80
B.	24	M	46	83	7	1.85	0.67	0.79	1.00	0.31
All Men					327	4.00	0.90	0.28	0.73	0.71

* Grouped according to children, women and men.

of 0.54. In men it was conductance = 0.28 (volume − 0.73 liter), with a correlation coefficient of 0.71. In individuals the correlation coefficients were higher than they are in this grouped data (Table I).

DISCUSSION

The results show that airway conductance increases when lung volume is increased by inspiration of air in an individual. When children are compared to adults, the larger individuals with larger lungs have higher airway conductance.

In men and women the regression line of airway conductance against lung volume intercepts the volume scale at a positive value at zero conductance. Insofar as it is justifiable to extrapolate a regression line this implies that airway resistance

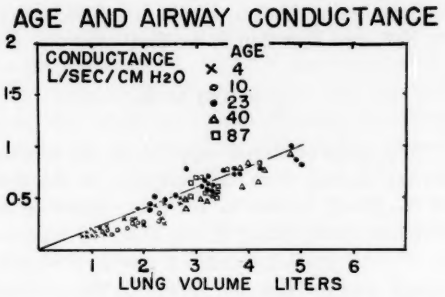

FIG. 7. THE RELATIONSHIP BETWEEN AGE AND AIRWAY CONDUCTANCE (LEFT) AND AIRWAY RESISTANCE (RIGHT) IN FIVE SUBJECTS EACH STUDIED AT VARIOUS LUNG VOLUMES

The major determinant of airway resistance is lung volume and age has little effect. The line expresses the approximate relationship conductance = ⅛ lung volume.

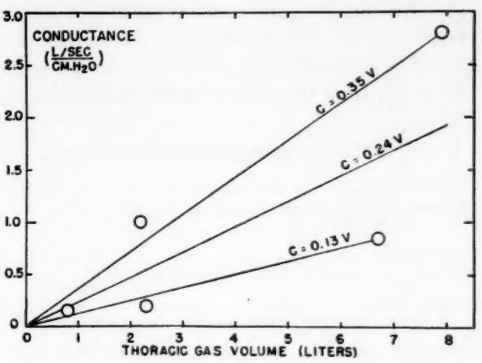

FIG. 8. POINTS WHICH INCLUDE THE OUTERMOST VALUES OF REGRESSION LINES IN FIGURES 4, 5 AND 6, AND THE LINES WHICH ARE ESTIMATED TO ALLOW APPROXIMATE PREDICTION, FROM THORACIC GAS VOLUME, OF THE UPPER LIMIT, MEAN, AND LOWER LIMIT OF AIRWAY CONDUCTANCE IN MOST NORMAL MEN, WOMEN AND CHILDREN

will become infinite before the lungs have completely emptied. This accords with the fact that lungs do not collapse completely on the autopsy table. The intercept in children, however, is negative although their lungs like those of adults do not collapse completely when the chest is opened. It seems therefore that these regression lines only apply over the range of lung volumes in which the observations were made. These ranges are indicated for each case in Figures 4, 5 and 6.

Airway conductance may increase as much as fourfold when an individual inflates his lungs from residual volume up to total lung capacity. This is somewhat greater than the estimate of 2.5 times by Mead and Whittenberger (4). Since the effect of lung volume on airway conductance and resistance is so marked, studies of changes in airway resistance after experimental or therapeutic procedures in individuals may be invalidated if concomitant changes in lung volume are not taken into account. Several methods of correction could be used but it seems best to use the slope of the regression line of conductance on lung volume in all adults studied. A sample calculation of this is given in the following paragraph.

In Figure 11 in the paper of Fry, Ebert, Stead and Brown (5), the mean value of Δconductance/Δalveolar volume in their three normal subjects can be calculated to be about 0.35. In our subjects this mean regression coefficient is 0.28. If conductance were initially 1.0 (resistance 1.0) and if as a result of a therapeutic procedure lung volume increased by 500 cc. then conductance would be expected to have increased by 0.14 and airway resistance would be expected to have fallen to 0.88. This change would have occurred in association with the change in lung volume and without the necessity of invoking any therapeutic effect of a bronchodilator.

It seems possible to use our data in tentative consideration of the order of magnitude of the relative distensibility of the lung as a whole in comparison to that of the resistance airways, *i.e.*, those airways which have the major part of the airway resistance of the whole lung. Consider a small spherical group of alveoli supplied by one resistance airway leading from the periphery to the center of this sphere.

Let V = lung volume in cc. and R = radius in cm. of the spherical mass of lung. Let l = length in cm. of the resistance airway in this sphere. C = conductance in cc. per cm. H_2O·second, and C′ = conductance in cm.^{5} per dynes second. Let r = radius of airway in cm., η = viscosity of air in dynes second per cm.^{2} and b = the regression of C on V. In the simplest case where the re-

gression line of C on V intercepts the V axis at zero [7]:

$$C' = \frac{bV}{981} \text{ from the regression line of C on V,} \quad 1$$

$$V = \frac{4}{3}\pi R^3 \text{ from the equation for a sphere,} \quad 2$$

$$C' = \frac{4\pi R^3 b}{3 \times 981} \text{ from } 1 \text{ and } 2, \quad 3$$

$$C' = \frac{r^4\pi}{8\,l\,\eta} \text{ from Poiseuille's equation.}^5 \quad 4$$

Let us assume that as the lung expands the airways lengthen in proportion to other linear dimensions related by a constant k:

$$l = k\,R, \quad 5$$

$$C' = \frac{r^4\pi}{8\,\eta\,k\,R} \text{ from } 4 \text{ and } 5, \quad 6$$

$$\frac{r^4\pi}{8\,\eta\,k\,R} = \frac{4\pi R^3 b}{3 \times 981} \text{ by equating } 3 \text{ and } 6, \quad 7$$

$$\frac{r^4}{R^4} = \frac{4 \times 8 \times bk\eta}{3 \times 981} \text{ by rearranging and cancelling,} \quad 8$$

whence

$$\frac{r}{R} = \sqrt[4]{\frac{4 \times 8 \times bk\eta}{3 \times 981}}. \quad 9$$

Since all the terms on the right are constant, the ratio r/R is also constant, in which case the radius of the resistance airways increases by the same fraction as the radius of a sphere of lung during inflation, *i.e.*, the resistance airways are as distensible as the alveoli.[8] This is in general agreement with the findings of Shepard, Campbell, Martin and Enns (8) on the changes in the dead space with lung volume as determined by single breath analysis.

[7] In the more complicated case where this is not so and where the regression line at zero conductance intercepts the X axis at a volume of several hundred cc., as in some of our subjects, the error in the considerations of dr/dR which follow is about 20 per cent. This is negligible when, as here, we are considering orders of magnitude.

[8] For purposes of this discussion, "distensibility" is defined as the fractional change of radius resulting from a given change in transpulmonary pressure (*i.e.*, mouth pressure minus intrapleural pressure). Expansion of the lung puts tension on the lung tissues, which presumably by traction lengthen and widen the air passages. Unfortunately, we do not know the magnitude and direction of these tension forces in the lung tissues.

Substituting known values, $b = 0.28$, $\eta = 1.98 \times 10^{-4}$, into Equation 9, we find

$$\frac{r}{R} = 0.03 \times \sqrt[4]{k}. \quad 10$$

In a group of alveoli supplied by one resistance airway leading from the periphery to the center of the group, k, and its 4th root, cannot be very different from unity. If our observed regression of C on V applied to such a group of alveoli, it would suggest that the radius of the resistance airway is about 3 per cent of the radius of the alveolar mass. In a lung composed of many such groups of alveoli arranged in parallel, our data would have the same meaning since parallel conductances and lung volumes can be added. Since the major part of the resistance is in the smaller airways (9) and since the relationship in Equation 10 is dependent not on k itself but on its relatively unchanging 4th root, it may be that this concept of small groups of alveoli each supplied by one resistance airway can in fact be applied to the whole lung.

Simplified formulas for predicting airway conductance and resistance

In deciding whether the airway resistance measured on a patient lies within normal limits, it is desirable to have a formula for predicting the mean and range of values for resistance which one may encounter on normal subjects. Such a formula should not include very unusual data, nor should it be more complicated than the known principles warrant. Unfortunately, statistics do not take some of these considerations into account. However, we may, by inspection, hope to derive some simple equations to include about 95 per cent of normal cases.

All regression lines of airway conductance on lung volume lay within an area on the volume-conductance graph bounded by the points (0.8, 0.15), (2.3, 0.20), (6.7, 0.85), (7.9, 2.8) and (2.2, 1.0). The range of normal is more simply delineated by an angle formed by points (7.9, 2.8), (0, 0) and (6.7, 0.85). This excludes the point (2.2, 1.0) obtained on a dwarf in full inspiration, and the point (2.3, 0.2) obtained on one subject, R. B., in full expiration, yet includes all the remaining data on men, women and children (it

even includes data on the dwarf and on Subject R. B. at ordinary breathing levels). These lines have the equations: $C = 0.13$ V and $C = 0.35$ V.

The mean predicted conductance for men and women can be obtained from the regression lines stated in the text, or can be simplified by inspection to: $C = 0.24$ V (range, 0.13 V to 0.35 V or ± 46 per cent). These points and lines are shown in Figure 8. The predicted airway resistance would then be: $R_A = 4.2/V$ (range, 2.9/V to 7.7/V). The borderline between normal and slightly abnormal is not at present sharply defined, and these equations should be applied with caution in questionable cases until further experience has been obtained.

CONCLUSIONS

Five hundred forty-six measurements of airway resistance and lung volume were made on 26 normal subjects of both sexes, with ages ranging from 4 to 87 years. In each subject studies were made at different degrees of inflation of the lung. Airway conductance, the reciprocal of resistance, was approximately linearly related to the degree of inflation of the lungs. Regressions of airway conductance on lung volume are given for children, women and men. Airway resistance in children is three to five times that of adults. This is related more to differences in lung volume than to differences in age. A method is suggested for correcting changes in airway resistance for concomitant changes in lung volume during studies on airway resistance. Theoretical considerations suggest that the small airways in which resistance to flow is located may be as distensible as the lung as a whole. A simplified method for predicting normal limits for airway conductance at different lung volumes is given.

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CHANGES IN IRON METABOLISM IN EARLY CHLORAMPHENICOL TOXICITY [1, 2]

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The frequency of hematopoietic depression occurring as a result of chloramphenicol toxicity is not known. Generally it is assumed that the incidence is low in relation to the total amount of chloramphenicol used (1). The deleterious action of chloramphenicol has been noted in all degrees of severity from fatal aplastic anemia (2–6), to minimal marrow depression readily reversible upon removal of the drug (7).

The clinical and laboratory signs of frank marrow hypoplasia with anemia, leukopenia and thrombocytopenia, attributable to chloramphenicol toxicity, are readily apparent. This advanced stage of toxicity bears a high mortality rate, and therefore early detection before irreversible changes transpire would be highly desirable.

This report is concerned with the early detection of erythropoietic depression due to chloramphenicol, by means of ferrokinetic techniques. Studies of iron metabolism included measurement of the rate of disappearance of tracer amounts of radioactive iron (Fe^{59}) from the plasma, determination of the subsequent appearance of radioactivity in the circulating erythrocytes and changes in the plasma iron content and plasma iron binding capacity. The results indicate that erythropoietic depression due to chloramphenicol has a higher incidence than aplastic anemia, and that such depression can be detected by ferrokinetic changes before abnormalities of the peripheral blood counts appear. Likewise, erythropoietic depression is detectable when there are no demonstrable changes in leukocytes, thrombocytes or bone marrow cellularity. Although it is a reasonable hypothesis that if chloramphenicol therapy were to be continued this depression limited to red cell production might be a sign of impending aplastic anemia, such an assumption remains unproven.

METHODS AND MATERIAL

Fifteen patients receiving chloramphenicol were studied. These included 9 females and 6 males, with ages ranging from 19 to 85 years; 11 patients were colored and 4 were white. Disease states for which chloramphenicol was administered included: infected traumatic and surgical wounds; meningitis; appendiceal abcess; urinary tract infections with and without uremia; pneumonitis; and infected leg ulcer. The dosage of chloramphenicol varied from 0.75 to 4 grams per day and duration of therapy ranged from 3 to 27 days (Tables I and II). In the majority of instances the patients were selected at random, but several cases were studied because of apparent hematopoietic changes.

The rate of removal of radioactive iron from the plasma and its subsequent incorporation into the erythrocytes was determined according to the method of Huff and associates (8). Ten microcuries (5 to 10 micrograms) of radioactive iron (Fe^{59}) [5] were incubated under sterile conditions with 20 milliliters of the subjects' heparinized plasma for 20 or 30 minutes and then administered intravenously. Blood samples were drawn at 15 to 30 minute intervals for the subsequent 2 to 4 hours and the radioactivity in the plasma counted in a well-type scintillation counter with a thallium activated NaI crystal.[6] Plasma counts of radioactivity were 10 to 20 times background. Subsequently, radioactivity appearing in the circulating erythrocytes was determined daily by counting whole anticoagulated blood which had been frozen and thawed in order to lyse the red cells and maintain a homogeneous mixture. External scintillation counting was performed over the liver, spleen, sacrum, heart and ribs in four patients after Fe^{59} administration, using the methods of Huff and associates (9).

In all the graphs, tables and discussion referring to the

[1] Presented in part at the meeting of the Central Society for Clinical Research, Chicago, Ill., November 1, 1957.

[2] This investigation was supported by a research grant (CY-1678c5) from the National Cancer Institute of the National Institutes of Health, Public Health Service, and was aided in part under A.E.C. Contract No. W-31-109-emy-78.

[3] American Cancer Fellow.

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[5] Fe^{59} was supplied by Abbott Laboratories in the form of ferrous citrate, 100 microcuries per milliliter and 48 micrograms per milliliter.

[6] NRD Instrument Co., Model cs-600.

TABLE I

Patients receiving chloramphenicol who did not manifest erythropoietic depression

Age, color, sex	Disease process	Bacterial organism	Chloramphenicol dosage	Duration of therapy
			Gm./day	*days*
1. 51 WF	Chronic pyelonephritis	*Coli-aerogenes*	1	15
2. 62 CF	Acute purulent meningitis	unknown	3	19
3. 53 CF	Appendiceal abscess	*Coli-aerogenes*	2	3
4. 36 CM	Infected traumatic wounds	*Staphylococcus aureus*	1	11
5. 20 WF	Chronic pyelonephritis	*Proteus vulgaris*	2	10
6. 19 CF	Fever of undetermined origin	unknown	2	10
7. 41 CF	Infected surgical wound	*Staphylococcus albus*	2	6
8. 83 CM	Pneumonia	unknown	1	12
9. 23 CF	Infected surgical wound	unknown	0.75	34
10. 60 CM	Lung abscess	*Proteus vulgaris*	2	27

patients with erythropoietic depression the individual case numbers refer to the same patient. In cases numbered 1, 3, 5, chloramphenicol was discontinued at the time of the first Fe^{59} study. Drug therapy was discontinued in case number 2, five days prior to the original Fe^{59} study. In case number 4, chloramphenicol administration was continued for 11 days after the initial radioactive studies, at which time the patient was discharged from the hospital and lost to further follow. She was restudied as soon as located two and one-half months later.

Measurements of plasma iron and plasma iron binding capacity were determined according to the method of Schade, Oyama, Reinhart and Miller (10) coincident with each radioactive iron study and additionally as indicated.

Sternal marrow aspirations were performed with a 16 gauge Osgood needle on each patient showing abnormalities of ferrokinetics. A nucleated cell count was performed and fixed sections were prepared from each marrow aspirate to aid in estimation of marrow cellularity.

Erythrocyte and leukocyte counts were performed in the standard manner and determination of packed cell volume was by the method of Wintrobe (1). Reticulocytes were counted by the wet method using brilliant cresol blue as stain. Platelets were ennumerated by a variant of the method of Dameshek (11), using 2 per cent sodium citrate as diluent.

RESULTS

In 10 of the 15 patients receiving chloramphenicol, ferrokinetic studies were either normal or compatible with the underlying disease process which was acute or chronic infection. The time elapsed for one-half of the Fe^{59} to disappear from the plasma (half time) varied from 22 to 98 minutes with appearance of significant amounts of radioactivity in the erythrocytes within 48 hours. The plasma iron values ranged from 16 to 100 micrograms per 100 millilters with saturation of the iron binding capacity varying from 5.5 per cent to 39 per cent (Table III). External scintillation counting performed in three patients revealed that as the Fe^{59} disappeared from the plasma major amounts of radioactivity were detected over the sacrum with lesser amounts counted over the liver and spleen.

The remaining five patients showed evidence of depression of erythrocyte production attributable to chloramphenicol. These five patients with erythropoietic depression received chloramphenicol in doses ranging from 1 to 4 grams per day for pe-

TABLE II

Patients receiving chloramphenicol who did manifest erythropoietic depression

Age, color, sex	Disease process	Bacterial organism	Chloramphenicol dosage	Duration of therapy
			Gm./day	*days*
1. 75 WM	Cystitis with bacteremia	*Proteus vulgaris*	4	11
2. 51 WM	Septicemia	*Escherichia coli*	3	20
3. 30 CF	Acute glomerulonephritis	*Coli-aerogenes*	2	7
4. 32 CF	Varicose leg ulcer	*Staphylococcus aureus*	2	15
5. 85 CM	Urinary infection with uremia	*Coli-aerogenes*	1	21

TABLE III

Ferrokinetic studies in patients receiving chloramphenicol without erythropoietic depression

	½ time of disappearance of Fe^{59} from plasma during therapy	Appearance of significant* amounts of Fe^{59} in red blood cells	Plasma Fe	Saturation of iron binding globulin
	min.	*hrs.*	*μg./100 ml.*	%
1.	37		77	38
2.	78		100	39
3.	85	48	16	5.5
4.	22		71	
5.	60	48	47	17.5
6.	98	48	89	31
7.	43	24	41	25
8.	27	24	26	16.5
9.	36	24		
10.	35	48	33.5	18.5

* Counts in the whole blood were at least 2 times background to be considered significant.

riods of 7 to 21 days. None had received chloramphenicol previously. Studies with Fe^{59} in these five patients showed a marked delay in the disappearance of radioactive iron from the plasma in four cases, with one-half times of disappearance ranging from 130 minutes to over 240 minutes (Table IV). The half time in the fifth patient was 98 minutes which falls in the range of normal (normal half times vary from 50 to 100 minutes in this laboratory), but significant amounts of radioactivity failed to appear in the red cells dur-

TABLE IV

Ferrokinetic studies during and following chloramphenicol administration in patients exhibiting toxicity

	½ time* of Fe^{59} disappearance from the plasma during therapy	½ time of Fe^{59} disappearance from the plasma after therapy was discontinued	Delay in appearance of significant radioactivity in the red cells
	min.	*min.*	*days*
1.† ‡	240	24	8
2.§	130	42	8
3.	260	50	9
4.‖ ¶	98	70	17
5.	145	85	11

* Normal ½ time is 50 to 100 minutes.

† In cases 1, 3 and 5, chloramphenicol was discontinued the day of the original Fe^{59} study.

‡ In cases 1, 2, 3 and 5 the second Fe^{59} study was performed when radioactivity first appeared in the erythrocytes.

§ In case 2 chloramphenicol therapy was discontinued five days prior to the original Fe^{59} study.

‖ Case number 4 received chloramphenicol for 11 days following the original Fe^{59} study.

¶ Case number 4 was restudied two and one-half months after the original Fe^{59} study.

TABLE V

External scintillation counting over various organs and radioactivity in the blood in chloramphenicol toxicity in case number 1*

Time following administration of Fe^{59}	Sacrum	Sternum	Liver	Blood
	cpm/μc.	*cpm/μc.*	*cpm/μc.*	*cpm/ml.*
15 min.	174	330	342	850
1 day	180	306	488	110
2 days	142	260	516	0
4 days	144	246	582	0
5 days	144	198	624	0
7 days	168	186	588	0

* Counts are expressed per microcurie of administered Fe^{59}.

ing the additional 11 days of chloramphenicol administration, confirming the presence of erythropoietic depression. In all five cases the disappearance of radioactivity from the plasma formed a

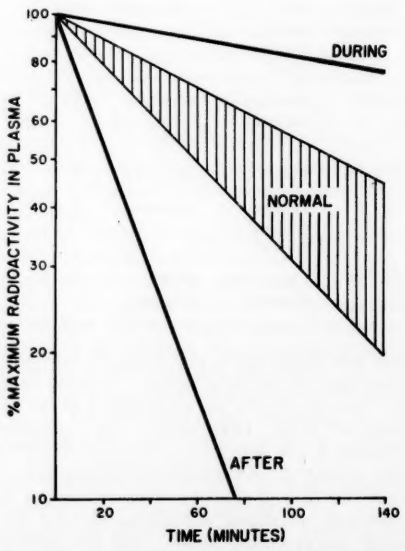

FIG. 1. THE EFFECT OF CHLORAMPHENICOL TOXICITY ON PLASMA DISAPPEARANCE OF Fe^{59}

The curves of Fe^{59} disappearance from the plasma in Patient number 1 with chloramphenicol toxicity are plotted with the logarithm of per cent maximum radioactivity on the vertical axis and time in minutes on the horizontal axis. The hatched area represents the range of normal curves of disappearance. The curve labeled "during" shows the markedly delayed disappearance at the time that chloramphenicol toxicity was detected. The rapid disappearance labeled "after" expresses the findings of a repeat study eight days after chloramphenicol was discontinued.

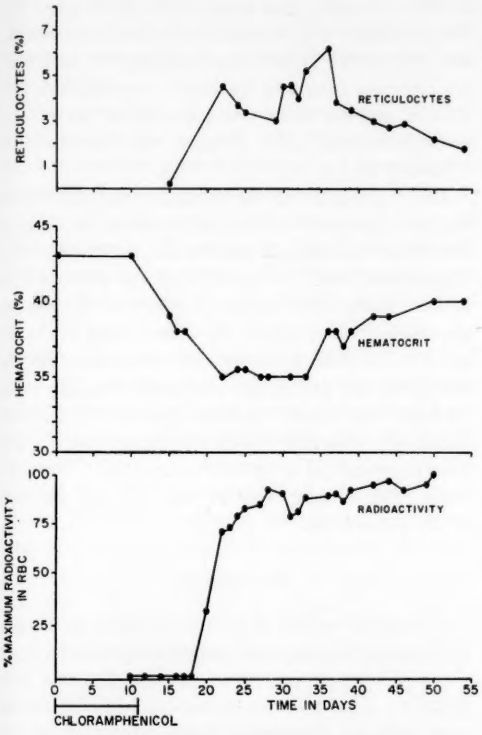

FIG. 2. EFFECT OF CHLORAMPHENICOL ON HEMATOCRIT, RETICULOCYTES AND APPEARANCE OF Fe^{59} IN RED CELLS

The data on Patient number 1 with chloramphenicol toxicity is shown on three graphs. Following administration of Fe^{59} no radioactivity appeared in the red cells for nine days. Maximum radioactivity appearing in the whole blood was 1,797 counts per minute per milliliter. Hematocrit was normal when toxicity was first detected, but subsequently dropped. A reticulocytosis was noted coincident with the appearance of radioactivity in the erythrocytes.

TABLE VI

Changes in hematocrit with chloramphenicol toxicity

Case	Hematocrit when toxicity discovered	Lowest hematocrit during toxicity	Hematocrit after recovery
no.	%	%	%
1	43	35	40
2	35	28	36†
3	20	20*	37
4	37	30.5	42
5	30	32*	34†

* Hematocrit maintained with blood transfusions.
† Continuing infection.

TABLE VII

*Changes in plasma iron and saturation of the iron binding globulin in chloramphenicol toxicity**

	Plasma iron* during therapy	Plasma iron after therapy was discontinued	Saturation of IBG† during therapy	Saturation of IBG after therapy was discontinued
	μg./100 ml.	*μg./100 ml.*	%	%
1.	222	30	86	11
2.	171	77	91	35
3.	71	26	53	12
4.	197	98	82	33
5.	161	49	100	17

* Normal plasma iron is 95 ∓ 25/100 ml. Normal saturation of the iron binding globulin is 28 per cent ∓ 2.
† IBG stands for iron binding globulin.

single exponential curve. External scintillation counting performed in one patient revealed that abnormally large amounts of radioactivity were to be found in the liver with lesser amounts counted over the sternum and sacrum (Table V).

Eight or more days after chloramphenicol ther-

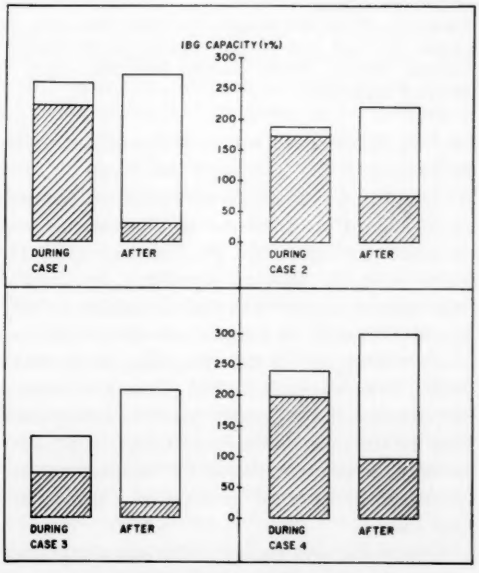

FIG. 3. SATURATION OF IRON BINDING GLOBULIN IN CHLORAMPHENICOL TOXICITY

The total height of each bar depicts the total iron binding capacity of the plasma, expressed in micrograms per 100 milliliters. The hatched portion in each case represents the plasma iron in the same units. The bar labeled "during" shows the findings during chloramphenicol toxicity, while that labeled "after" represents the measurements when definite recovery was noted.

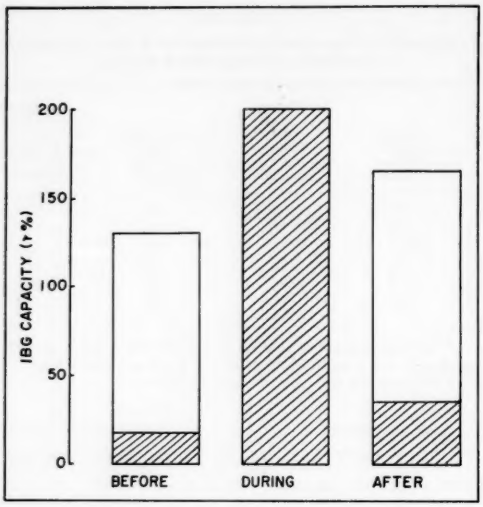

FIG. 4. CHANGES IN IRON BINDING GLOBULIN IN CHLORAMPHENICOL TOXICITY

The plasma iron data in Patient number 5 with chloramphenicol toxicity is depicted in the same manner as in Figure 3. From left to right the three bars show the plasma iron and iron binding capacity before chloramphenicol therapy, during toxicity, and after signs of recovery appeared.

apy was discontinued, repeat studies of plasma disappearance of Fe^{59} revealed half times of 24 to 84 minutes, which values either fall in the range of normal, or are shortened as is commonly seen in infection (Figure 1). For this same period of eight or more days no significant amounts of radioactivity appeared in the circulating erythrocytes. Normally significant amounts of radioactivity will appear in the circulating erythrocytes within 24 to 48 hours, and 85 per cent or more of the administered radioactivity will be incorporated into the red cells within 7 to 10 days. Characteristically the incorporation of Fe^{59} into the erythrocytes will be hastened in acute or chronic infection (12).

One of the five patients with chloramphenicol toxicity had hypoplasia of the bone marrow with anemia, leukopenia and thrombocytopenia; however, the other four cases had no leukopenia or thrombocytopenia, and bone marrow aspiration and section revealed normal or increased cellularity. Two patients were not anemic when depressed red cell production was detected, but manifested a subsequent fall in hematocrit during the period of toxicity and nonproduction (Figure 2). Two patients had azotemia and marked anemia, and further fall in hematocrit during the period of toxicity was obscured by blood transfusions. In all five patients there was recovery of hematocrit towards normal after therapy was discontinued (Table VI).

Determinations of the plasma iron pattern in the five patients with erythropoietic depression correlated with the alterations observed with Fe^{59}. Thus, there was an elevation of the plasma iron in four cases with values of 161.5 to 222 micrograms per 100 millilters. However, in all five cases the iron binding globulin was abnormally highly saturated, the percentage saturation ranging from 53.3 per cent to 100 per cent (Table VII, Figures 3 and 4). Normal values of plasma iron in this laboratory are 95 ∓ 25 micrograms per 100 milliliters, with a saturation of iron binding globulin of 28 per cent ∓ 2.[7]

DISCUSSION

The abnormalities of iron metabolism in the five patients exhibiting chloramphenicol toxicity were those of complete suppression of erythrocyte production. These were: an elevation of the plasma iron with an abnormally high saturation of the plasma iron binding globulin (13); a delay in the disappearance of radioactive iron from the plasma (14–16), with external scintillation counting in the one patient so tested revealing abnormally large amounts of radioactivity deposited in the liver rather than in the organs normally containing bone marrow (9); failure of the radioactivity to appear in the circulating red corpuscles (15) for a period of eight or more days; and a decrease in the volume of packed red cells.

These abnormalities of iron metabolism cannot be attributed to infection alone. Bacterial infection without chloramphenicol toxicity characteristically gives the following findings: a lowered plasma iron with decreased saturation of the iron binding globulin (13); a rapid disappearance of radioactivity from the plasma with external counting indicating that the Fe^{59} appears in the bone marrow as it is cleared from the plasma; and a prompt appearance of radioactivity in the erythrocytes (12). Infection alone, without chloram-

[7] Normal values were established in 20 healthy medical students studied in the morning, fasting state.

phenicol administration, has not been observed to produce a pronounced depression of red cell production, even in cases of overwhelming and fatal bacteremia.

Measurements of the disappearance of Fe^{59} from the plasma coupled with knowledge of the plasma iron and plasma volume may be utilized to calculate the plasma iron turnover (8). The resultant value is usually indicative of the rate of erythropoiesis. However, in aplastic anemia and other states associated with nonproduction of erythrocytes, verified by the failure of radioactivity to appear in the circulating erythrocytes, the plasma iron turnover may be normal or elevated (14, 16). It has been suggested that under these circumstances the plasma iron turnover cannot reflect the rate of effective erythropoiesis, but rather is indicative of the exchange of iron between the plasma and the iron stores (14). In the absence of any correlation between the plasma iron turnover and erythropoiesis, such as occurs in chloramphenicol toxicity, any calculation of plasma iron turnover will be misleading rather than informative. Accordingly, the ferrokinetic data is presented here in terms of the times in which one-half of the administered radioactivity disappeared from the plasma. With the exception of hemachromatosis, a marked prolongation of the one-half time of Fe^{59} disappearance is characteristic only of states in which erythropoiesis is suppressed. The failure of radioactivity to appear in the red cells is confirmatory and provides proof of decreased erythrocyte production, in those instances of delayed disappearance of Fe^{59} from the plasma.

Some agents such as certain antimetabolites, alkylating agents and gamma radiation have been shown invariably to cause hematopoietic depression and bone marrow aplasia if administered in adequate quantities; while others such as the 5-substituted hydantoins, the straight chain analogs of the hydantoins, and the 2,4, dione ring structures will cause only occasional and unpredictable marrow depression or aplasia. Chloramphenicol is to be found in this latter group of agents whose toxic action has been labeled "drug idiosyncrasy" (1). Previously recognition of hematopoietic depression due to chloramphenicol has depended on observing depression of leukocyte and thrombocyte counts in the peripheral blood accompanied by the morphologic changes of hypoplasia or aplasia of the bone marrow. It has been established that bone marrow function is not necessarily correlated with bone marrow morphology (1). Disparity between an actively cellular marrow and a severe depression of erythrocyte production was a prominent feature in four of the five cases exhibiting chloramphenicol toxicity, in whom there was no apparent disturbance in marrow cellularity, leukocytes or thrombocytes. In these four cases it was only by means of Fe^{59} studies and determination of the changes in the plasma iron pattern that the failure of erythrocyte production could be verified. Particularly in two of these patients (Numbers 1 and 4), in whom the degree of anemia was mild and chloramphenicol was discontinued because the infection was eradicated, there would have been no reason to suspect chloramphenicol toxicity since the bone marrows were highly cellular and leukocyte and platelet counts were normal.

It remains unknown whether these four patients would have acquired hypoplasia of the bone marrow had chloramphenicol therapy been continued, or whether a period of reversible erythropoietic depression may be an isolated toxic manifestation and be unrelated to depression of leukocytes and platelets. However, in the absence of data to the contrary it is a reasonable hypothesis that the reversible depression of red cell production is a manifestation of the same toxic action of chloramphenicol that can produce irreversible and often fatal aplasia of the bone marrow. If the latter concept is true then factors of duration of therapy, dosage of chloramphenicol, individual patient sensitivity or other unknown factors may be incriminated. In any event it is reasonable to assume that early detection of chloramphenicol toxicity may prevent more serious consequences.

In regard to early detection of erythropoietic depression due to chloramphenicol it is noteworthy that changes in plasma iron pattern correlated with the abnormalities of ferrokinetics. In each case, at the time the Fe^{59} studies demonstrated supression of erythrocyte production, the plasma iron binding globulin was abnormally highly saturated with iron, with a return to lower levels of saturation when recovery occurred. The high degree of saturation of the iron binding globulin did not occur in the other patients with infection except when an obvious hemolytic episode was present. It would appear that determination of

the plasma iron and plasma iron binding capacity provides a simple means of detecting early toxicity in patients receiving chloramphenicol and that these determinations would be useful in screening other agents with potentially toxic effects on the bone marrow.

SUMMARY AND CONCLUSIONS

1. Fifteen patients receiving chloramphenicol were studied utilizing ferrokinetic methods and determinations of the plasma iron pattern. Five of these patients exhibited severe erythropoietic depression attributable to chloramphenicol; however, this does not represent a true incidence.

2. One of the five patients with erythropoietic depression had leukopenia, thrombocytopenia and hypoplasia of the bone marrow; however, the other four cases had no discernible abnormality of leukocytes or thrombocytes, and bone marrows showed normal or increased cellularity.

3. In two cases with chloramphenicol toxicity, abnormalities of iron metabolism were observed to precede the onset of anemia.

4. Abnormalities of ferrokinetics were: a delay in the disappearance of Fe^{59} from the plasma; appearance of radioactivity in the reticulo-endothelial system rather than in the bone marrow; and failure of radioactivity to appear in the circulating erythrocytes for eight or more days.

5. Coincident with the observation of abnormalities of ferrokinetics, the plasma iron binding globulin was noted to be abnormally highly saturated with iron. With recovery from toxicity the saturation decreased.

6. The abnormalities of iron metabolism noted in chloramphenicol toxicity are readily discernable from the changes seen in infection alone.

7. Determination of the plasma iron pattern provides a simple means of detecting erythropoietic depression. Such early detection may well be instrumental in preventing the more serious consequences of aplastic anemia due to chloramphenicol or other drugs with potentially toxic effects on the bone marrow.

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THE RELATIONSHIP OF THE AGE OF RABBIT ERYTHROCYTES TO THE EFFECTS OF INOSINE ON THEIR OSMOTIC RESISTANCE [1, 2]

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Several recent publications have described physical, chemical and biological differences between young erythrocytes, including reticulocytes, and mature erythrocytes of man and other species. Reticulocytes and young erythrocytes of the rabbit have been shown to contain more potassium and sodium, to have a greater cell volume and to have a lower density than the older cells (2). Differential centrifugation and radioiron labeling have been employed to demonstrate the increase in density with *in vivo* ageing of human erythrocytes (3, 4), and it was noted that after centrifugation the cells of the lower layers contained less water, potassium, chloride and bicarbonate (5). Differences in electrolyte composition had been observed previously on analyzing separate layers of human erythrocytes obtained on centrifugation (6). Further evidence has been provided that rabbit reticulocytes have an intact tricarboxylic-acid cycle and cytochrome system which are not present in mature erythrocytes (7). Increased cholinesterase activity has been demonstrated in the reticulocyte-rich upper layer of cells of centrifuged blood of rats, rabbits and man (8, 9), and a decline in cholinesterase and catalase activity with *in vivo* ageing of human erythrocytes has been observed (10). Immature erythrocytes of rabbits (2) and man (11, 12) appear to be more resistant to hemolysis in hypotonic media than are the older erythrocytes. Diminished glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase activities have been found in osmotically sensitive human erythrocytes as compared to these enzymatic activities in the osmotically resistant younger cells (11).

In studies on the relationship of metabolic activity of the erythrocyte to its structural integrity, the resistance of fresh human erythrocytes to osmotic stress has been found to be enhanced after incubation of the erythrocytes with certain purine nucleosides (13). This phenomenon was associated with a considerable uptake and further metabolism of the ribose moiety of the effective purine ribosides (14). The present report is concerned with the influence of *in vivo* ageing of rabbit erythrocytes upon the effect of the purine riboside, inosine, on osmotic resistance. Glycine-2-C^{14} was administered to a series of rabbits and, at intervals during the life span of the isotopically labeled erythrocytes, the effect of inosine on the osmotic resistance of the erythrocytes of different ages was determined.

MATERIALS AND METHODS

Adult male white New Zealand rabbits weighing 4.5 to 5.0 Kg. were given three subcutaneous injections, totalling 10 ml. over a two to three hour period, of glycine-2-C^{14} in isotonic sodium chloride solution containing 0.032 mM per ml. with a specific activity of 625 μc. per mM. At intervals of 4, 8, 46 and 60 days after injection one of the rabbits was heparinized and exsanguinated, and the blood was collected in acid-citrate-dextrose solution (Cutter Laboratories, National Institutes of Health Formula B) in the proportion of four volumes of blood to one volume of acid-citrate-dextrose. The blood was centrifuged immediately and the plasma and buffy coat were removed. The erythrocytes were washed three times in isotonic sodium phosphate buffer, pH 7.3, with centrifugation. Twenty-two per cent sus-

[1] This work was presented in part at the Forty-Ninth Annual Meeting of the American Society for Clinical Investigation at Atlantic City, N. J., May 6, 1957 (1).

[2] The work of this laboratory, of which these studies form a part, has been supported by grants from the Office of Naval Research [Contract Nonr-1765 (00)], the National Heart Institute of the National Institutes of Health, United States Public Health Service (Grant H-2803), the American Cancer Society, and the Atomic Energy Commission [Contract AT(30-1)1855].

[3] Fellow of the National Foundation for Infantile Paralysis, 1955–1957.

pensions of the thrice washed erythrocytes were incubated either in isotonic sodium phosphate buffer alone or in buffer containing inosine in a final concentration of 15 micromoles per ml. of suspension for two hours at 37° C. Following incubation, the erythrocytes were washed twice and subjected to hypotonic lysis by the method previously described (13), except that 16 ml. aliquots of each suspension were added to 64 ml. portions of sodium phosphate buffer (pH 7.3) solutions of varying hypotonicity and to distilled water. Hypotonic solutions were so selected that the extent of lysis in the control and inosine-treated erythrocyte suspensions was comparable. The supernatant hemolysates were aspirated carefully after centrifugation, and the extent of hemolysis was calculated from the amount of hemoglobin liberated as measured by the optical density at 541 mμ determined in a Beckman DU spectrophotometer and related to the distilled water sample whose hemolysis was taken as 100 per cent.

Duplicate portions of the hemolysates were diluted ten or fifteen times with unlabeled hemoglobin obtained from washed erythrocytes of rabbits of the same strain. A total of 2 to 3 Gm. of hemoglobin was precipitated by the addition of the hemolysates diluted with carrier hemoglobin to two and a half volumes of acetone. This dilution procedure was found to be accurate to within about 3 per cent. Hemin was extracted from the precipitated hemoglobin with three portions (100, 75 and 50 ml.) of 1.2 per cent hydrochloric acid in acetone with centrifugation and filtration by a modification of the Anson and Mirsky procedure (15). Hemin was isolated in crystalline form by methods previously reported (16) and known to yield hemin of constant specific activity upon repeated crystallization. The radioactivity of the hemin (29 ± 1 mg.) was determined at infinite thickness in teflon planchets with a center well 1 cm. in diameter in a Nuclear-Chicago gas flow counter with a micromil window, model No. D-47. The counting error did not exceed 4 per cent.

RESULTS AND DISCUSSION

The influence of the age of rabbit erythrocytes upon their resistance to osmotic lysis is reflected

TABLE I

The influence of the age of rabbit erythrocytes on 1) osmotic resistance and 2) the effect of inosine on osmotic resistance *

Phosphate control			Inosine treated		
Per cent of isotonicity 1	Per cent hemolysis 2	Specific activity† 3	Per cent of isotonicity 4	Per cent hemolysis 5	Specific activity† 6
		4 days after glycine-2-C^{14}			
44	34	701 ± 4	42	35	500 ± 2
43	43	820 ± 6	41	40	544 ± 4
42	51	925 ± 20	40	50	638 ± 12
41	57	938 ± 12	39	58	740 ± 15
40	66	1,018 ± 38	38	69	868 ± 12
0	100	1,285	0	100	1,325 ± 15
		8 days after glycine-2-C^{14}			
38	33	1,118 ± 1	36	33	795 ± 0
37	40	1,185 ± 15	35	38	836 ± 11
36	46	1,295 ± 15	34	43	898 ± 28
35	55	1,375 ± 5	33	51	968 ± 12
			32	57	1,082 ± 22
0	100	1,655 ± 30	0	100	1,720
		46 days after glycine-2-C^{14}			
43	34	810 ± 10	41	40	845 ± 25
42	46	810 ± 30	40	48	895 ± 5
41	53	805 ± 15	39	53	905 ± 15
40	60	865 ± 25	38	61	910 ± 30
39	67	865 ± 25	37	69	900 ± 0
0	100	1,040	0	100	1,072
		60 days after glycine-2-C^{14}			
43	32	1,312 ± 8	41	36	1,420 ± 10
42	38	1,245 ± 15	40	48	1,315 ± 15
41	48	1,225 ± 5	39	54	1,340 ± 10
40	59	1,215 ± 35	38	62	1,305 ± 5
0	100	1,360	0	100	1,388

* Extent of hemolysis in hypotonic phosphate buffer and specific activity of hemin from lysates of rabbit erythrocytes incubated for two hours at 37° C. in isotonic sodium phosphate buffer alone or with 15 micromoles inosine per ml. of incubation suspension.

† Counts per minute above background per standard planchet (29 ± 1 mg. hemin) corrected for dilution with carrier hemoglobin ± deviation from the mean.

in the data of Table I, Columns 1 to 3. At any one time after administration of glycine-2-C^{14}, random lysis of erythrocytes in the various hypotonic phosphate buffer solutions would have yielded hemin of the same specific activity regardless of the extent of hemolysis, *i.e.*, the radioactivity of the hemin isolated from the hemoglobin of the lysed erythrocytes would have been the same as that observed in an aliquot of the whole suspension as represented by the 100 per cent hemolysis samples. Actually, however, in the experiments performed four and eight days after injection of the labeled glycine, the specific activity of the hemin at points between 33 and 66 per cent hemolysis was only 55 to 83 per cent of that of the whole suspension. It is apparent, therefore, that the erythrocytes four or eight days of age and younger were more resistant to hypotonic lysis than the average of the total cell population.

In the experiment performed with the erythrocytes from the rabbit that received glycine-2-C^{14} 46 days earlier, the specific activity of the hemin from those cells which were lysed at various levels of hypotonicity was slightly less than that of the 100 per cent hemolysate. Therefore, these labeled cells were still somewhat more resistant to hypotonic lysis than the average of the whole cell population. After 60 days, the specific activity of the hemin at various degrees of lysis was similar to that observed at 100 per cent lysis.

These data demonstrate increased osmotic resistance of young erythrocytes when compared to the average of the entire cell population, and they are consistent with the findings reported recently from other laboratories (2, 11, 12). The specific activities observed in the experiment at 60 days indicate that the cells of this age have a susceptibility to hemolysis essentially similar to that of the average of the total cell population. If these older cells have an increased susceptibility to lysis, one might expect, in the 60 day experiment, slightly greater specific activity in the hemin samples isolated from hemolysates in which the extent of hemolysis is less than 100 per cent, since at 60 days the erythrocytes are nearing the end of the expected life span of rabbit erythrocytes (17). It would be desirable, therefore, to investigate the osmotic resistance of old erythrocytes at ages greater than 60 days and at levels of lysis in which only a small fraction of the cells are destroyed.

In every instance, under conditions of comparable osmotic stress, the extent of hemolysis of inosine-treated erythrocytes was less than that of control erythrocytes (Table I, Columns 1, 2, 4 and 5). The finding that inosine enhances the osmotic resistance of rabbit erythrocytes is similar to that previously reported for human erythrocytes (13).

In the hemin isolated from hemolysates obtained from erythrocytes incubated with inosine four and eight days after the rabbits received labeled glycine, the specific activity was lower than that of the hemin of the control hemolysates at comparable levels of lysis (Table I, Columns 4 to 6). Figure 1 presents the ratios of the specific activity of the hemin from the inosine-treated erythrocytes to the specific activity of the hemin from the control erythrocytes at 40, 50 and 60 per cent hemolysis as interpolated from curves of specific activity plotted against per cent hemolysis. If no preferential protection of isotopically labeled erythrocytes on incubation with inosine had occurred, these ratios would have been equal to unity. In the experiments performed with erythrocytes from rabbits injected with glycine-2-C^{14} four and eight days earlier, the ratios were 0.70

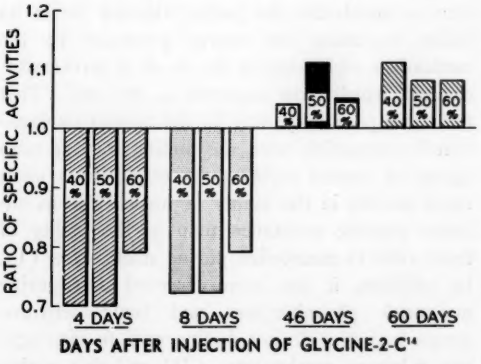

FIG. 1. RELATIONSHIP OF THE AGE OF RABBIT ERYTHROCYTES TO THE EFFECT OF INOSINE ON THEIR OSMOTIC FRAGILITY

$$\text{Ratio} = \frac{\text{Specific activity of hemin from lysates of inosine-treated erythrocytes}}{\text{Specific activity of hemin from lysates of control erythrocytes}}$$

Per cent refers to per cent hemolysis in hypotonic phosphate buffer solutions after incubation in isotonic sodium phosphate buffer alone or with inosine for two hours at 37° C., as interpolated from curves of specific activity plotted against per cent hemolysis.

to 0.79. It is apparent, therefore, that rabbit erythrocytes four or eight days old and younger were preferentially protected against hypotonic lysis by incubation with inosine. After 46 and 60 days the ratios were 1.04 to 1.11, findings which indicate that no preferential protection of the labeled cells had occurred. The ratios slightly greater than unity could conceivably be the result of some increased hemolysis of the labeled erythrocytes incubated with inosine, but more likely reflect the protection by incubation with inosine of the young, unlabeled erythrocytes.

The findings with inosine-treated rabbit erythrocytes are compatible with other observations that the effects of incubation with inosine are dependent upon metabolic activity of the erythrocyte. These effects include enhanced resistance to osmotic lysis (13, 18), increased organic phosphate esters within the cells (18), restoration of intracellular potassium (18, 19) and sodium concentrations (20), and perhaps slight prolongation of viability of *in vitro* stored erythrocytes (18, 21, 22). The decline in the effect of inosine on the osmotic resistance of rabbit erythrocytes which have aged normally *in vivo* may indicate either a concomitant decline in the ability of the erythrocyte to metabolize the purine riboside or in its ability to utilize the energy produced by the metabolism of inosine as the result of irreversible changes which have occurred in the cell. That the latter possibility may be the correct explanation is compatible with the finding that *in vitro* ageing of human erythrocytes results in a more rapid decline in the ability of nucleosides to enhance osmotic resistance than in the ability of these cells to metabolize purine nucleosides (1). In addition, it has been observed that purine nucleoside phosphorylase and lactic dehydrogenase activities do not decline with *in vivo* ageing of human erythrocytes (23) and that purine nucleoside phosphorylase activity does not decrease in the erythrocytes surviving prolonged *in vitro* storage (24).

SUMMARY AND CONCLUSIONS

On labeling of rabbit erythrocytes *in vivo* with glycine-2-C^{14} it was demonstrated that: *1*) Young erythrocytes are more resistant to hypotonic lysis than the average of the whole cell population, and *2*) incubation with the purine nucleoside, inosine, for two hours at 37° C. in isotonic sodium phosphate buffer, pH 7.3, increases the resistance to osmotic stress of young erythrocytes much more than that of the average of the total cell population.

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THE HIGH SPECIFICITY OF THE MANGANESE PATHWAY THROUGH THE BODY[1,2]

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The accepted ideas about the physiological role of manganese have been derived predominantly from *in vitro* experiments. However, these are characterized by lack of specificity: Only a limited discrimination between manganese and some other metals is shown, for instance, in metal transport systems (1, 2), in enzyme activation (2–4) and in the preservation of mitochondria (5). The conclusions from these experiments were of interest because they threw new doubt on the functional specificity and even on the essentiality of manganese in the living organism. Thus it became necessary to ascertain whether manganese may be replaced in the intact animal as well. To investigate this, we have used a variation of the classical technique of flooding the organism with metals which we were led to believe would substitute for manganese. Surprisingly, these kindred metals were ineffective in eluting radiomanganese from the body: Only manganese compounds proved effective in that regard.

The results suggest that there exists a segment in the pathway of manganese through the body, the properties of which permit the passage of that metal only. This paper deals with the implications of this finding both in reference to manganese as well as to the elements which have been thought not to possess such an *in vivo* specificity.

MATERIALS AND METHODS

Experiments with intact animals

Animals. These were seven week old Swiss albino male mice, weighing 16 to 18 grams. They were housed in cages, the bedding of which consisted of shredded corn cobs. When dietary restrictions were imposed, metal screening was used to prevent the animals from eating the bedding. Regardless of the type of bedding, the furs remained radiologically clean, as shown by the failure of Versene® and detergent baths to lower the total body radioactivity.

Diets. Since the manganese turnover depends upon dietary intake, an effort was made to keep the manganese intake constant. In the bulk of the experiments, the same lot of Purina Fox chow was used ($MnSO_4$ reported by manufacturer as 0.02 per cent). As a "manganese free" diet, a vitamin B_{12} deficient rat diet was used.[3] To this a salt-vitamin fortification mixture[4] was added without supplementary manganese, unless otherwise stated.

Stable metals and their salts. Rhenium dioxide was prepared by dissolving the metal in nitric acid and heating to dryness with anhydrous hydrazine HCl. The procedure was repeated twice with the precipitate. This was finally washed with water, ground into a fine suspension in normal saline and injected into the animals intraperitoneally. All the metals, oxides and salts[5] used in these experiments to challenge the isotope were injected with normal saline as the vehicle. In some experiments (see Table I) the sulfates or chlorides of the metals were dissolved with added equimolar amounts of sodium citrate (hereafter referred to simply as "citrate"). In those latter experiments the controls received equimoler sodium citrate instead of the routinely used saline injections. The doses of the challenging materials ranged from 1×10^{-4} to 1×10^{-5} mole of metal. These were given either as single or as daily injections for one week.

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[2] A preliminary report of this paper was presented befor the Federation of American Societies for Experimental Biology in Chicago, March, 1957.

[3] Nutritional Biochemicals Corporation. The complete diet contained 2.5γ of manganese per 100 gram diet (or 1γ of manganese per gram ash).

[4] Nutritional Biochemicals Corporation, "Vitamin Diet Fortification Mixture."

[5] Spectrographic tests of the $MnSO_4$ powder showed it to be free from contaminating metals, while both the $FeSO_4$ and the $CrCl_2$ powders contained 0.01 per cent manganese. The utilized chemical agents and their sources of purchase are as follows: cobaltous sulfate, ferrous chloride, manganese dioxide, nickelous sulfate and sodium citrate from Baker and Adams Co.; copper sulfate, magnesium sulfate, potassium permanganate and zinc sulfate from Baker's; ferric chloride from Merck & Co.; manganous chloride from Mallinkrodt; manganese metal from A. D. Mackay; vanadyl sulfate from Fisher; and vanadium metal from Amend Drug. The rhenium compounds were obtained from the University of Tennessee except for the rhenium pentachloride (K & K Labs.).

Isotopes. Mn^{52} ($T/2 = 5.7$ days) was prepared by the Cr^{52} (d, 2n) Mn^{52} reaction in a 60 inch cyclotron by one of us (J. J. G.). Separation of the tracer Mn^{52} from the chromium necessitated a precipitation with manganese carrier. When the latter was found to have a marked influence on the phenomena under study, this preparation was abandoned in favor of Mn^{54}. The latter isotope was prepared carrier free by the Nuclear Science and Engineering Corporation. It was received as $Mn^{54}Cl_2$ in hydrochloric acid (200 μc. per ml.). Neutral dilutions (0.8 and 1.6 μc.) were given intraperitoneally to the animals in 0.1 or 0.2 ml. of saline. While with the Mn^{52} variations even from 0.1 to 0.2 μc. brought upon concordant variations of the turnover rate, the Mn^{54} preparation was free from such complicating effects. Accidental injections into a hollow viscus were soon followed by almost complete loss of the animal's radioactivity.

Measurements of the radioactivity. Each tagged animal was placed in a 50 ml. plastic centrifuge tube with its mouth opposite to the outlet of an oxygen line. After plugging with cotton, the tube was inserted into the horizontal well of a Texaco® counter. The same procedure (minus the ventilation) was used for pooled organs, to permit the direct comparison between animals and their organs. As a rule counting was accurate to within 1 per cent in both cases, and its geometry was constant between 3 and 30 ml.

Calculations of turnover rates. The animal's total body radioactivity was counted within one hour after isotope injection. This was repeated daily, in some cases for as long as 20 to 30 days. The subsequent counts were expressed as percentages of the first, after being corrected for background and, if needed, for isotopic decay. The data were then plotted on semilogarithmic paper as a function of time and the curves were drawn (Figure 1). When necessary, these were analyzed graphically for their constituent components (Figure 2) by subtracting the straight end from the remainder of each curve (6). In the experiments in which challenging injections of carrier metals were given, the following controls were used: *1*) saline- or sodium citrate-challenged animals; *2*) each animal's own prechallenge turnover rate; *3*) animals that had received $MnCl_2$ in amounts often less than equimolar to that of the challenging metal. At the end of some experiments, in which the challenge did not effect the turnover rate, the isotope's continued availability for exchange was demonstrated by injecting $MnCl_2$ solutions.

Summary of typical protocol. Thirteen mice were injected intraperitoneally with 1.0 μc. of $Mn^{54}Cl_2$ in 0.2 ml. saline, and their total body radioactivity determined. They were then divided into four groups. The retained radioactivity was measured at daily intervals. The corrected counts were plotted semilogarithmically against time as per cents of the first count. On the third day, Group I received 1×10^{-5} mole of $CrCl_2$; Group II, 0.2 ml. of saline; Group III, 2×10^{-7} $MnCl_2$; Group IV, 1×10^{-5} $FeCl_2$. The day following the injection the phenomenon illustrated in Figure 4 was seen. It was followed for five days and then the 2×10^{-7} mole of $MnCl_2$ were given to the animals that had received $CrCl_2$ and $FeCl_2$. This was done in order to test whether their tracer had remained exchangeable. The animals were then sacrificed, and the peritoneal cavities were found to be free of metallic deposits.

OBSERVATIONS

Turnover of body manganese. The results shown in Figure 1 were obtained with a group of nine Mn^{54} tagged animals. These animals were maintained on Purina Fox chow. The individual data fell into exceedingly smooth curves so that. subsequently, breaks in the curves which were experimentally induced could be used as additional controls. When these curves were graphically analyzed, at least two components were found ($T/2 = 48$ to 68 hours and $T/2 = 230$ to 300 hours).

Two groups of three mice each were given 1 per cent ammonium chloride and 1 per cent sodium bicarbonate instead of drinking water. After no effect on the turnover was seen, their bottles were switched. This again failed to produce any change in the turnover rates.

Effect of diet on manganese turnover. The feeding of the "manganese-free" diet to a group of three mice a few days prior to and during the experiment did not effect the rate of the first component ($T/2 = 54$ hours), while that of the second increased to $T/2 = 860$ hours. The diet was enriched with carrier manganese sulfate (0.01 per cent of Mn^{++} w/w), and the procedure was repeated in three other mice. This showed turnover

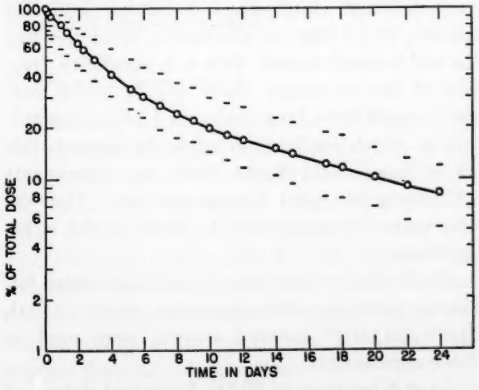

FIG. 1. TOTAL BODY TURNOVER OF Mn^{54} IN MICE (SEE TEXT)

The dashed lines define the envelope of all the data.

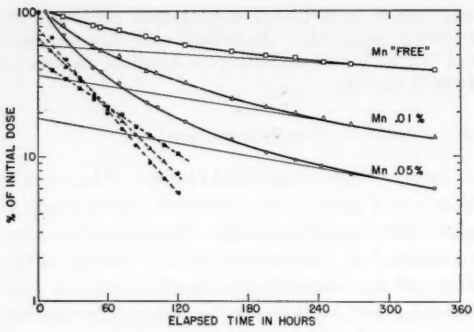

FIG. 2. TOTAL BODY TURNOVER OF Mn^{54} AS INFLUENCED BY DIETARY MANGANESE

rates of $T/2 = 38$ and $T/2 = 238$ hours for each of the two components. Increase of the manganese supplement to 0.05 per cent caused further decreases of these numbers to $T/2 = 31$ and $T/2 = 208$ hours in another group of three mice. Starvation of three animals markedly decelerated the turnover. Obstruction of the intestine at the anus in another three mice stopped the turnover entirely.

These experiments show that the rate of radiomanganese loss from the body is sensitive to the level of dietary manganese and to the state of gastrointestinal function. They are compatible with intestinal excretion of this metal (*7*). Concordantly, the acceleration of the observed turnover by added dietary manganese might have had the following implications: *1*) that more carrier manganese is absorbed from the gut than hitherto suspected and elutes the radioisotope from the tissues; or *2*) that on elimination from the gut, the stable metal carries with it a significant fraction of the bile-bound tracer (6, *7*) which normally might have been reabsorbed (8). Regardless of which explanation might be correct, this uncertainty would throw doubt on experiments with any stable metal, if these were fed. Thus the oral route was abandoned in favor of the intraperitoneal.

Accelerated elimination of radiomanganese following challenge with manganese carrier. Both Mn^{54} and Mn^{52} injected animals were used in these experiments.

As can be seen in Table I, various forms of the stable metal were given at various times. The valence state of the carrier ranged from 0 in the metal powder to 7 in the permanganate. Injections of all of these compounds were followed by acceleration of the rate of turnover, which resulted in losses ranging from 15 per cent to 80 per cent within twenty-four hours. Figure 3 illustrates two characteristic experiments at two different postinjection times with two different isotopes. By varying the time at which the

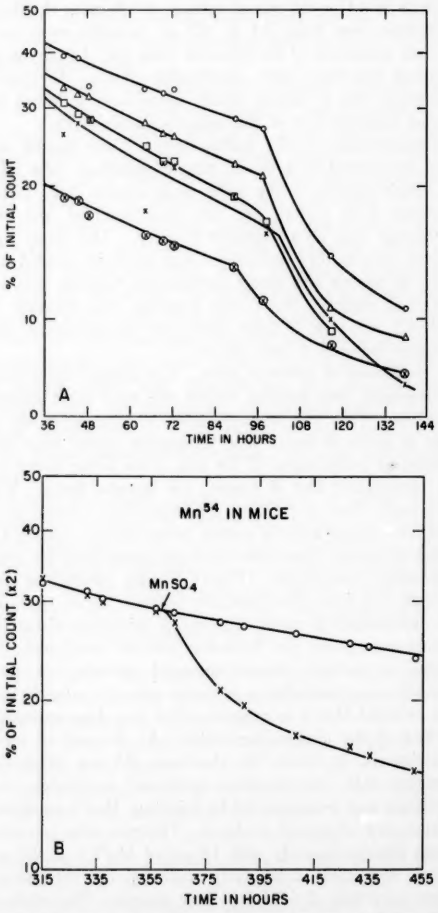

FIG. 3. EFFECT OF ADDING CARRIER MANGANESE ON RADIOACTIVE MANGANESE TURNOVER

A = Total body turnover of Mn^{52} in mice receiving 8×10^{-6} mole of carrier manganese as $MnSO_4$ at the 86th hour of the experiment.

B = Averages of total body turnovers of Mn^{54} in three mice compared with those of three mice receiving 4×10^{-6} mole of carrier manganese as $MnSO_4$ at the arrow.

challenge was made, it was found that an appreciable fraction of the tracer had remained exchangeable, even three weeks after tagging the animals. The exchange has not been quantitated as yet. The impression was gained, however, that elution of the tracer manganese was more obvious when the challenge was made early or with high doses of carrier. An easily observed acceleration of the turnover was evident following injections of 9×10^{-8} mole of $MnSO_4$ as late as the sixth day. The extreme valence states (Mn^0 and Mn^{7+}) seemed less effective than Mn^{2+}.

These experiments proved that the radioisotope remained largely and continuously available for elution by carrier. The elution was obvious after challenges with $MnSO_4$ amounting to 30 per cent of the estimated manganese content of the body.

Failure of some other stable metals to affect Mn^{54} turnover. Manganese is a member of both the first transition group and of group VIIB of the elements. Many of the members of the transition group (in periods four and five) have been known to substitute for manganese *in vitro* (1–5, 9). Manganese and rhenium are the only stable members of group VIIB. Rhenium is Mendeleef's celebrated dvi-manganese (10). His ekamanganese (technetium), which would have been very crucial in these studies, has only radioactive isotopes.

These metals were used as eluting agents in experiments identical with those described in the case of carrier manganese. Magnesium, the element most widely used *in vitro* instead of manganese, was also included. In another experiment, a member of group VIIA was tested because of its possible interchange with manganese in the thyroid (11).

The upper limit of the concentration in the challenging injections was set by the toxicity of the individual compounds. Safe doses were chosen after preliminary experimentation with groups of mice. Care had to be taken to avoid interference with the animals' eating and bowel habits which were known to affect the end point.

The lower limit was set at 30 per cent of the mouse's total body content of each of the stable metals used as eluting agents. This value was chosen because of the effect of carrier manganese described above. Furthermore, 30 per cent of anything appeared to be a large amount. Table I shows both the metals' estimated body content and the challenging doses given. High values were intentionally chosen in making these estimates. A mouse weighing 20 grams was chosen as a prototype in spite of the fact that the experimental animals weighed 16 to 18 grams. Even so, the desired value of 30 per cent was reached or exceeded by the individual injections. It was markedly surpassed in the experiments in which the injections were given daily for one week.

Every effort was made to compare similar valence states with each other. Some animals which had received toxic doses of ferrous and chromous chlorides had small peritoneal metallic desposits at autopsy. The mouse peritoneum had shown a high absorptive capacity in the experiments with manganese metal and dioxide powders. In spite of this the precaution was taken of including citrate in many of these injections, since this markedly facilitates absorption by forming chelates with these metals (12). The metals' release in the body was expected because citrate is rapidly metabolized.

These stable metals were incapable of affecting in any way the rate of elimination of radiomanganese from the body, in spite of all the specifications listed above. Figure 4 illustrates the failure of ferrous and chromous citrates to bring upon any change of the radiomanganese turnover in concentrations 50 times higher than an effective amount of manganous sulfate. That the isotope was available for exchange in these animals also was shown by the effect of the manganous salt at the end of that observation.

Experiments with organs

Following the observation that challenges with carrier manganese had caused elution of radiomanganese readily, reproducibly and regardless of the state of the carrier, the failure of any of the other stable metals to do so seemed incongruous. The following explanation for the apparent incongruity was considered and tested: It was thought that the nonmanganese metals might have caused elution of the tracer from one organ into another instead of elimination out of the body. This would not have been detected by our method, since it was designed to quantitate the radioactivity of the entire body and not to differentiate be-

TABLE I

Summary of metals administered to manganese^{54} injected mice

No. of mice	Metal or salt	Atomic number	Moles per 20 Gm. mouse*	Moles per challenge	$\Delta t\ \bar{p}$ isotope
7	$MgSO_4$	12	4.2×10^{-4}†	$5 \times 10^{-5} - 5 \times 10^{-4}$	146
3	$VOSO_4$	23	1.3×10^{-7}‡	1.0×10^{-5}	310–505§
9	Cr Cit	24	3.1×10^{-7}‡	$2 \times 10^{-6} - 1 \times 10^{-5}$	(−)6–505§
13	$CrCl_2$	24		$4 \times 10^{-6} - 1 \times 10^{-5}$	50–650
3	Mn^0	25	2.8×10^{-7}‡	$9 \times 10^{-5} - 4.5 \times 10^{-4}$	6–146
11	Mn Cit	25		$1.8 \times 10^{-6} - 9 \times 10^{-6}$	(−)6–674§
28	$MnSO_4$	25		$9 \times 10^{-8} - 1 \times 10^{-5}$	50–650
3	MnO_2	25		1.1×10^{-4}	(−)24
3	$KMnO_4$	25		2.5×10^{-6}	354
6	$FeCl_2$	26	2.1×10^{-5}‖	$9 \times 10^{-6} - 1 \times 10^{-5}$	50–650
11	Fe^{++} Cit	26		1.8×10^{-6}	(−)6–480§
3	Fe^{+++} Cit	26		1×10^{-6}	1
3	$FeCl_3$	26		3×10^{-6}	48
6	Co Cit	27	9×10^{-8}‡	$1.7 \times 10^{-6} - 8.5 \times 10^{-6}$	311–480§
3	$CoSO_4$	27		3.5×10^{-7}	266
3	Ni Cit	28	6.1×10^{-7}‡	$1.7 \times 10^{-6} - 8.5 \times 10^{-6}$	311–480§
7	Cu Cit	29	6.3×10^{-7}‖	$1 \times 10^{-7} - 3 \times 10^{-7}$	(−)6–364§
19	$CuSO_4$	29		$1 \times 10^{-7} - 6 \times 10^{-7}$	50–318§
3	Zn Cit	30	1.6×10^{-5}‡	$1.5 \times 10^{-6} - 7.6 \times 10^{-6}$	311–480§
3	$ZnSO_4$	30		3.5×10^{-7}	266
3	KIO_3	53	7.1×10^{-8}†	4.6×10^{-6}	354
7	Re^0	75		5.5×10^{-5}	6–50
7	ReO_2	75		4.6×10^{-6}	(−)24–270§
3	Re_2O_7	75		9.7×10^{-6}	1
3	$NaReO_4$	75		2.7×10^{-5}	354

* These are estimates of the mouse's total body content prior to challenge. All estimates pertain to the individual metal involved in the test and not to the salt listed.
† Estimated from Hawk, Oser and Summerson (14).
‡ Estimated from Tipton and co-workers (18).
§ Include daily injections for one week.
‖ Estimated from Underwood (9).

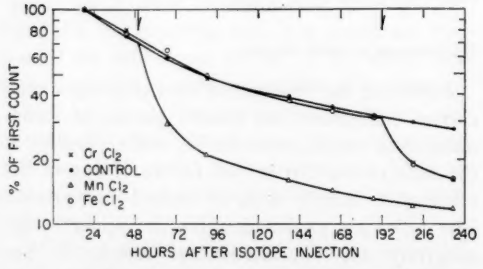

FIG. 4. TOTAL BODY TURNOVER OF Mn^{54} IN MICE AS INFLUENCED BY SOME CARRIER METALS

At first arrow 0.2 ml. of saline, 1×10^{-5} mole of either $FeCl_2$ or of $CrCl_2$, or 2×10^{-7} mole of $MnCl_2$ were injected in each of the groups. The 2×10^{-7} mole of $MnCl_2$ were injected, at the second arrow, to the $FeCl_2$ and $CrCl_2$ treated animals.

tween its parts. Thus we investigated the effects of some of these stable metals on the partition of the tracer amongst the organs of the body. The intracellular organelles could be also tested from that standpoint, particularly since it was observed earlier (13) that the mitochondria concentrate manganese isotopes. For this, one might have chosen an organ on the basis of some gross observation: As will be seen below, no such lead was forthcoming.

In view of the size of the enterprise involved in using all of the previously tested metals in this study as well, we had to arrive at a compromise: We chose elements 24, 25 and 26 (Cr^{++}, Mn^{++} and Fe^{++}).

For brevity, only Experiments A and B (Fig-

ure 5) are described out of four concordant ones (the others differed in several experimental details). In both of these the animals were divided into groups of three and eight, respectively, injected with radiomanganese tracer and tested for radioactivity on the first day. In Experiment A these groups were injected with 1×10^{-5} mole of sodium citrate or manganous, ferrous or chromous citrates per animals, respectively. The injections were made both in the morning and the evening of the second day. On the third, they were sacrificed within the hour following the test of their radioactivity. The ferrous citrate animals had given the impression of toxicity by displaying lesser physical activity and rougher furs than the controls. Thus, in Experiment B, the number of mice per group was increased, the manganous citrate group was omitted, the challenging dose of ferrous citrate was reduced to 2×10^{-6} mole and all the carriers were injected on the second and third days. The animals were sacrificed on the fourth day and were handled as in Experiment A.

The bodies of the animals were dissected and their organs divided as shown in Figure 5. The organs of each group were pooled and tested for radioactivity. Careful inspection of the peritoneal surfaces showed no metallic deposits.

As is shown in Figure 5, the only appreciable effect on the distribution of the tracer amongst the organs tested was again brought about by the manganese preparation. The others yielded a distribution comparable to that of the controls.

This phenomenon had the characteristics of the effect we had planned to show with the other stable metals, namely elution from one to another part of the body. It occurred above and beyond the accelerated elimination induced by manganese: In the experiments with the dissected organs, we have reported only the radioactivity retained in the body.

DISCUSSION

The uncertainty of projecting facts from the test tube into the realities of integrated life is clearly illustrated by this study. At the outset one was led to expect that several metals might elute radiomanganese from the body. Such expectation was based on the following facts: *1*) Many of the *in vitro* substitutes for manganese

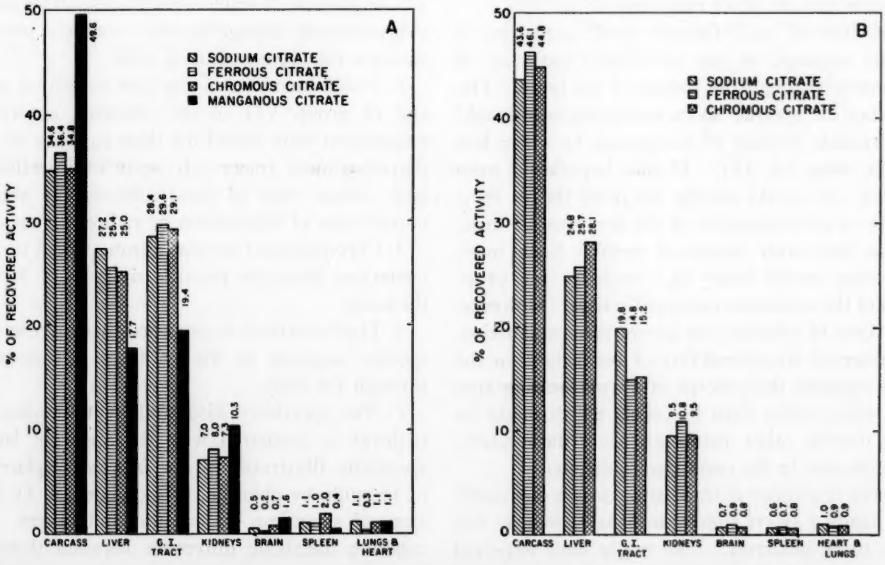

FIG. 5. PARTITION OF RADIOMANGANESE AMONG TISSUES OF MICE FOLLOWING INJECTION OF STABLE METAL COMPOUNDS

A and B summarize the results of Experiments A and B, respectively, described in the text.

bind more strongly with tissue constituents (2). *2*) Some are abundant, like magnesium, while the trace concentrations of manganese might not suffice to satisfy all its postulated reactions (14). *3*) The known *in vitro* transport mechanism does not guard against competitors (1).

A review of the coordination chemistry of manganese (2, 12) did not reveal any one single property which alone could explain these results. If *all* the known coordination properties are considered, however, this specificity can indeed be easily explained. Therefore, one is justified to assume that there exist in the body micro-compartments possessing many properties, the sum total of which determine the entrance of manganese and manganese only. These might include: a fairly resilient octahedral arrangement of six charges; oxygen as the predominating donor atom; a space with a radius of 0.5 to 0.9 Å. Such an environment would discriminate effectively against the entrance of other metals. If one also assumes that a given redox potential prevails at such a site, then specific reactions would be favored. A redox potential of minus 1.33 volts, for instance, would favor the reaction $Mn^{++} + H_2O = MnO_2 + 4H^+ + 2E^-$, while it would discriminate against other reactions.

The sum of such factors would constitute a specific segment in the anatomical pathway of manganese through the tissues of the body. The existence of specific atom configurations would not preclude binding of manganese by other less specific sites (2, 12). If this hypothesis were granted, one could ascribe many of the *in vitro* results to oversaturation of the few specific manganese sites with excess of metal. Such oversaturation would bring into evidence the properties of the numerous nonspecific sites. However, regardless of whether one favors this explanation, the observed irreplaceability of manganese in the body supports the concept of its performing specific tasks, rather than functions which might be taken over by other metals whenever the randomness inherent in the mass-law so dictates.

There is another extrapolation which one might have made *a priori* which also was shown by this work to be incorrect. One might have expected that manganese would be displaced by some metal (other than manganese) because of such well-known precedents as the displacement in the body of bromide by chloride (15), of molybdenum by tungsten (16) and of strontium by calcium (17). There exists, however, at least one metabolic difference between manganese and the elements listed above: the route of excretion. Manganese is excreted in the feces, while the others (Br, Mo, Sr, and so forth) are excreted primarily in the urine. This suggests that a search for other common differences might be rewarding. Furthermore it is obviously doubtful that manganese is the only substance whose pathway is strewn with specific segments, since one would expect similar behavior from iron also. With this in mind, an investigation of the pathways of other elements is now in progress.

SUMMARY

1. A procedure is described which permits observation of the elution of radiomanganese by injected stable metal compounds in intact mice and their tissues. This delineates an area for direct correlations between *in vivo* and *in vitro* phenomena.

2. Stable manganese compounds ranging from powdered metal to permanganate were effective eluting agents of the body's radiomanganese.

3. Injections of stable manganous citrate caused a pronounced change in the isotope's partition amongst the organs of these mice.

4. Stable members of the first transition group and of group VII of the elements as well as magnesium were tested for their capacity to elute the manganese tracer. In spite of injections of large doses, none of these affected the animal's normal rate of elimination of radiomanganese.

5. Chromous and ferrous citrates failed to cause deviations from the normal partition of Mn^{54} in the body.

6. The hypothesis is proposed that there exists a specific segment in the pathway of manganese through the body.

7. The specificity displayed by the manganese pathway is compared with the apparent lack of specificity illustrated by the *in vivo* displacement of bromide by chloride, of molybdenum by tungsten, of strontium by calcium and others. One common metabolic difference between these elements and manganese is noted: the route of excretion. This is entirely fecal for manganese and primarily renal for the other elements cited.

ACKNOWLEDGMENTS

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THE CLEARANCE OF HETEROLOGOUS PROTEIN FROM THE CIRCULATION OF NORMAL AND IMMUNIZED MAN [1, 2]

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The clearance rates of intravenously administered antigenic protein from the blood stream of normal and immunized animals have been studied by a number of observers and the results have been reviewed by Coons (1). Proteins labeled in various ways were used during these investigations, but the most relevant results appear to have been obtained when proteins were trace-labeled with radioisotopes (2), a technique resulting, under the best conditions, in little or no detectable change in the biological fate of the protein. In most instances the experimental conditions were such that the rate of protein removal was gradual and the period of observation extended over several days. However in one recent study (3) particular attention was paid to the changes occurring in the first hour or so after the injection of the antigenic material into the immune animal, a time during which antigen-antibody union in the circulation might be expected to exert a marked influence upon the clearance rate. Virtually all previous investigators have used the rabbit as a test animal and studies in man have not been reported.

Streptokinase, a product of hemolytic streptococcal metabolism, when injected into man by the intravenous route and at appropriate dosage, will cause the activation of serum plasminogen and the occurrence of a fibrinolytic state (4). During the course of work aimed at inducing a state of controlled fibrinolysis in man for therapeutic reasons (5), the opportunity arose to determine the plasma clearance rates of streptokinase from the circulation of normal and immune man. Most streptococcal proteins are antigenic to man and streptokinase is no exception; consequently as a result of naturally occurring hemolytic streptococcal infections the population exhibits a variable level of specific antibody toward this material. Indeed, the injection of streptokinase will induce in almost all, if not all, subjects and after a latent period a rapid sustained rise of specific antibody. Despite the antigenic nature of streptokinase the intravenous injection of highly purified preparations (6) is tolerated without clinical or other evidence of deleterious effect (7).

It is perhaps a misnomer to talk of unimmunized man in connection with streptokinase as this is an ubiquitous antigen; probably few humans constitute virgin soil with regard to it. Nevertheless, this term is used to indicate those persons who show either very low or no circulating levels of specific antibody. Moreover, one outstanding advantage accrues from the use of this system since it enables studies to be carried out on a population which has been subjected to an antigenic stimulus of a "normal" intensity, rather than a population that has received an intense "unphysiological" stimulus. Thus the mechanisms disclosed may be pertinent to the problems encountered under human disease conditions. Furthermore, in all previous studies, doses of several mg. antigen N per Kg. body weight have been used, whereas in this study it has been possible to use only a few micrograms antigen N per Kg. body weight. The use of these much smaller doses might be expected to yield results more immediately relevant to the course of events following the common types of immunological insult in man.

The present communication describes the findings following the intravenous administration of isotopically labeled streptokinase into normal (*vide supra*) and immunized man. The results indicate that the elimination of antigenic protein from the blood stream of man parallels those mechanisms

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[2] Presented before the Thirtieth Annual Meeting of the Central Society for Clinical Research, Chicago, November 1 and 2, 1957.

described as occurring in the rabbit, though certain significant species differences do exist.

METHODS

Two methods were used to prepare streptokinase to be used for labeling. Material prepared by the first method (6) assayed at 600 streptokinase units per microgram N was biophysically homogeneous, but showed the presence of several antigenic components on immunochemical analysis; material prepared by the second method [3] was of similar potency and similarly homogeneous, by biophysical examination, but was markedly superior to the other on immunochemical analysis (8). Both materials reacted similarly to iodination procedures and both were treated identically following *in vivo* injection. The majority of the work was performed using material prepared by the second method.

The labeling technique was based on that of Eisen and Keston (9), the reaction pH being 8.8. An ion exchange column (Amberlite® IRA-400) was used to remove uncombined iodine. The eluate was taken to 50 per cent ammonium sulphate saturation and the precipitate dissolved in normal saline containing 0.05 M phosphate buffer at pH 7.5 with 1 per cent human serum albumin. Sterilization was by Seitz filtration (Swiney model), the pad being saturated by 1 per cent human albumin prior to use and being washed with 5 ml. of 1 per cent albumin after the streptokinase had been passed.

The streptokinase recovery after the resin stage was 85 to 100 per cent of the original activity, but usually a 10 to 30 per cent loss occurred during filtration as a result of the small amount of protein being handled (1 mg.). The amount of free I^{131}, as determined after trichloracetic acid precipitation, was under 3 per cent. The integrity of the iodination bond was tested by running paper electrophoretic strips and preparing radioautographs. The labeled material ran with the same mobility as unlabeled streptokinase and the radioautographs could be superimposed on the protein strips. Control determinations with I^{131} mixed with streptokinase showed the radioactivity to travel independently of the protein band and labeled material mixed with free I^{131} showed two bands on the radioautographs. The degree of I^{131} tagging varied with different preparations, but assuming a molecular weight of 60,000 for streptokinase (6) the degree of iodination produced in the batches used for human tracer studies varied from 0.2 to 0.7 atoms of I^{131} for each molecule of streptokinase. Thus the streptokinase used was trace-labeled rather than converted into heavily iodinated protein, a step which would have resulted in spurious clearance rates (10). It is apparent, assuming that the molecular weight for streptokinase is correct, that one atom of iodine may be substituted into each streptokinase molecule without loss of biochemical activity. However data on the effect of a higher degree of iodination are lacking. More heavily iodinated batches usually lost biochemical activity, but the effect was inconstant and was probably attributable to the differing chemical treatment of the proteins, rather than to the alteration of their structure.

Radio-activity was determined in a well-type scintillation counter with an efficiency of approximately 50 per cent. Blood samples withdrawn for assay were oxalated, centrifuged and the plasma counted. The protein was then precipitated by adding 2 ml. of 15 per cent trichloracetic acid to 3 ml. of plasma and centrifuging off the precipitate. Control runs with unbound I^{131} added to the plasma showed that over 95 per cent could be recovered in the supernatant and that iodinated protein was totally precipitable. All plasma counts, except where otherwise noted, refer to protein bound activity rather than to total plasma activity and all counts were corrected back to the original volumes. Clearance studies were made with 4 to 15 microcuries of activity. So as to facilitate comparisons between clearance studies performed with different batches of labeled material of varying radioactivity, clearance plots have been expressed as streptokinase units per ml. plasma, rather than as cpm per ml. plasma. Counting of centrifuged red cell concentrates revealed that no greater activity was found than could be accounted for by the entrapped plasma content and that this activity could be removed by washing. No increase of free I^{131} was found after labeled streptokinase had been incubated with blood or plasma for 12 hours at 37°C.

Streptokinase was assayed as previously described (6) and the biochemical activity expressed in Christensen units (11). Streptokinase antibody assays were performed upon the isolated gamma globulin fractions of serum prepared by the technique of Lever and co-workers (12) and assayed by a modification of the Christensen technique (11). Clearance studies were performed on patients who required intravenous streptokinase for therapeutic reasons. Assay samples were withdrawn from the arm other than the one that had been used for the streptokinase injection or infusion.

Relation of the specific antibody content of plasma to its total streptokinase inhibitory power. The dose of streptokinase required to induce whole blood fibrinolysis in man can be predicted by means of a simple *in vitro* test (the predicted dose test) that provides a measure of the total inhibitory effect of plasma against streptokinase (13). This figure, expressed in units streptokinase per ml. plasma, when multiplied by the patient's calculated plasma volume indicates the total streptokinase dosage to produce fibrinolysis. The inhibitory action of plasma upon streptokinase is a consequence of two mechanisms. First, since antiplasmin will inhibit plasmin, a threshold streptokinase concentration must be exceeded before detectable plasminogen activation occurs and second, as a consequence of immunization the plasma may contain streptokinase antibody.

It has been necessary to establish the relative importance of these two mechanisms in our patients at different levels of plasma inhibitory action; otherwise the use of a figure for total plasma antibody would have had only

[3] Kindly supplied by Dr. J. Ruegsegger, Lederle Laboratories, Pearl River, N. Y.

TABLE I

The antibody content of plasma related to the total inhibitory effect of plasma upon streptokinase

Total inhibitory effect of plasma on streptokinase units / ml	Average and range (in %) of inhibition due to antibody content of plasma	No. of observations
0–10	51 (37–68)	7
10–20	74 (58–97)	8
20–200*	86 (70–121)	7

* The techniques are less accurate at the highest levels of inhibition. As the degree of plasma streptokinase inhibition increases the greater is the proportion of inhibition contributed by specific antibody.

doubtful validity. Table I shows the comparison between the total inhibitory effect of the plasma (predicted dose test) and the fraction resulting from the presence of antibody. It is apparent that not only is the concept of the total plasma antibody valid, but that antibody accounts for the major part of the inhibition shown by our patients and that this mechanism is of increasing importance as the total inhibitory power of the plasma increases. Furthermore the occurrence of fibrinolysis must indicate that an excess of antigen has been infused.

Throughout this communication the value for total plasma antibody has been derived by multiplying the antibody content per ml. of plasma by the plasma volume calculated from the patient's body weight, and the demonstration of plasma or whole blood fibrinolysis accepted as evidence of antigen excess in the plasma.

Nature of the streptokinase-antibody complex. Specific antigen-antibody precipitates were never demonstrable despite the use of many immune sera and the trial of many antigen/antibody ratios, nor was isotopically labeled streptokinase precipitated by immune sera. Probably the failure to demonstrate precipitating antibodies rests on a quantitative rather than a qualitative basis, since calculation indicates that the quantity of streptokinase antibody found, even in hyper-immune sera, may be below the amount required to yield a detectable specific precipitate. However recent work using gel diffusion techniques suggests that precipitating antibodies against streptokinase may be demonstrable in both immune animal and immune human sera (8). Despite this latter finding it is clear that under the experimental conditions used, the antigen-antibody complexes formed were, at least *in vitro*, always of the soluble variety. Presumably a similar situation obtained *in vivo*.

RESULTS

Plasma clearance studies in man

Figure 1 (A) illustrates the rate of labeled streptokinase clearance from the circulation of a 56 year old man who received 100,000 units over a five minute period by the intravenous route. *In vitro* testing had shown him to have total plasma antibody equivalent to 29,000 streptokinase units. The rate of initial clearance is seen to be extremely rapid, but following the removal of a portion of the labeled material the clearance rate becomes much slower. Figure 1 (B) shows a precisely similar experiment in a 42 year old man, except that *in vitro* testing had shown him to have a total plasma antibody equivalent to 61,000 units. The curves in Fig. 1 (A) and (B) are seen to be of a similar form but to differ in respect to their proportions. Clearly a greater amount of the injected material has been removed rapidly in the latter experiment where the amount of circulating antibody was the greater. Analysis of these curves by a regression method (3) shows that each is composed of two exponential clearance components, the 50 per cent clearance rates for the first component being respectively 17 and

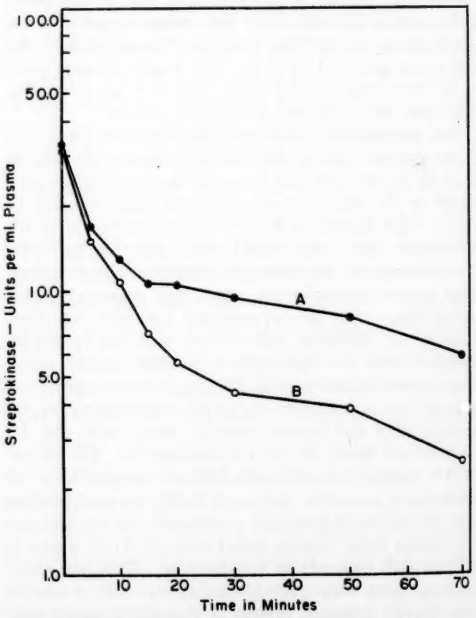

FIG. 1. THE PLASMA CLEARANCE RATE OF LABELED STREPTOKINASE

The plasma streptokinase concentrations were obtained after the injection, at zero time, of labeled streptokinase (100,000 units) in Patients A and B. The total plasma antibody, determined before the injection of streptokinase, was 29,000 units in Patient A and 61,000 units in Patient B.

TABLE II

Rate of streptokinase clearance from the circulation at various antigen/antibody ratios *

ANTIGEN DEFICIENCY			ANTIGEN EXCESS		
Total circulating antibody	Antigen/Antibody (in %) injected prior to clearance study	50% plasma clearance in minutes	Total circulating antibody	Antigen/Antibody (in %) injected prior to clearance study	50% plasma clearance in minutes
29,000	complex curve**	11	29,000	complex curve**	62
61,000	complex curve**	17	61,000	complex curve**	83
1,000,000	5	14	200,000	150	60
150,000	30	20	30,000	150	87
70,000	70	15	120,000	130	70
50,000	60	36	50,000	170	81
90,000	20	9	50,000	900***	92
65,000	90	15	80,000	1,000***	101
			65,000	1,400***	110

* Antibody assays were made prior to streptokinase therapy. Clearance studies during conditions of antigen excess (right half of the table) were made using less than 10,000 units of labeled streptokinase.

** Derived from data of Figure 1.

*** Clearance rates determined during the second day of therapy.

11 minutes and the 50 per cent clearance rates for the second component being 62 and 83 minutes. Since all streptokinase clearance curves performed under conditions of antigen excess show these two components, the first component has been designated as "fast" and the second as "slow." These designations employed throughout the text have been expressed as 50 per cent plasma clearance rates in minutes.

Though regression analysis of complex curves provides much information, it was felt to be preferable to study simple exponential clearance rates since the requisite information could be obtained with fewer analyses and thus fewer samples would have to be withdrawn from the patient. Since the fast rate of plasma clearance appeared to occur when circulating antibody was either in excess or equivalence with the antigen, six clearance studies were performed on individual patients utilizing less antigen than was required to combine with the total plasma antibody. In all instances the material was cleared at a simple exponential rate with an average 50 per cent clearance time of 17.5 minutes. Details of six experiments and the two earlier experiments are shown in Table II and the actual experimental findings for two patients are illustrated in Figure 2. It is to be noted that whatever the initial level of plasma antibody and whatever the proportion of injected antigen in relation to this last figure, the clearance rate remained approximately the same and, provided that antibody remained in excess, antigen clearance was very fast.

Unlabeled streptokinase in a dose at least 30 per cent in excess of that required to combine with all the circulating antibody was injected into seven patients. Brisk fibrinolysis was induced in all and following the production of this state, labeled material was injected and its plasma clearance determined. Two of the patients used were those whose fast rates of clearance are illustrated in Figure 2 and the results of the second clearance determination in these patients are also illustrated in Figure 2. Of the remaining five patients, the second clearance was determined in three during the second day of therapeutic treatment after they had received a large excess of antigen. The details of these seven clearance determinations are shown in Table II; the average 50 per cent rate of clearance under these circumstances was 83 minutes. The two rates of clearance, the fast and the slow, differ significantly (t equals 13.7 and $p < 0.001$) and the only explanation for this difference appears to lie in the presence of circulating antibody in the plasma during the determination of the first clearance rate and its absence during the determination of the second rate.

Evidence from long duration streptokinase infusions

Calculation from the data of Table II shows that to maintain a steady streptokinase concen-

tration of 10 streptokinase units per ml. plasma during the "slow" clearance phase approximately 30,000 streptokinase units per hour should be infused. However this calculation being based on short term plasma clearance studies probably offers inadequate correction for antigen removal by antibody union in the extravascular spaces. Accordingly, four patients whose total plasma antibody varied between 35,000 to 70,000 units received a dose of labeled streptokinase 50 per cent in excess of their total antibody. Commencing 30 minutes later, when the "fast" rate of clearance would be largely complete, further labeled streptokinase was administered at the rate of 50,000 units per hour for 8 hours. Serial determinations of plasma streptokinase concentrations were made during and after the infusions. Three of the four patients showed gradually rising plasma streptokinase concentrations peaking at 8 to 10 streptokinase units per ml. at the end of the infusion, but the fourth patient showed a sharper rise, his highest concentration being 18 units per ml. Thus in all the patients, during the eight hour period, the elimination rate of streptokinase from the circulation was less than, but not much less than, 50,000 streptokinase units per hour. This compared well with the calculated figure of 30,000 units per hour. However, one further patient who had a much higher total plasma antibody, 150,000 units, showed different behaviour. The experiment was conducted similarly except that he received 100,000 units per hour; his plasma streptokinase levels rose more slowly than those of the other patients and peaked at 6 units per ml. This difference was attributed to extravascular antigen fixation. Concomitant biochemical studies showed that in each patient the plasma streptokinase concentration was correlated with plasma fibrinolytic activity measured on preformed I^{131} labeled plasma clots (5).

Experience with the infusion of unlabeled streptokinase (65 infusions in 40 patients) has provided confirmatory evidence of our findings with labeled streptokinase. Empirically we have found that patients with an initial plasma antibody below 50,000 units maintain a relatively steady

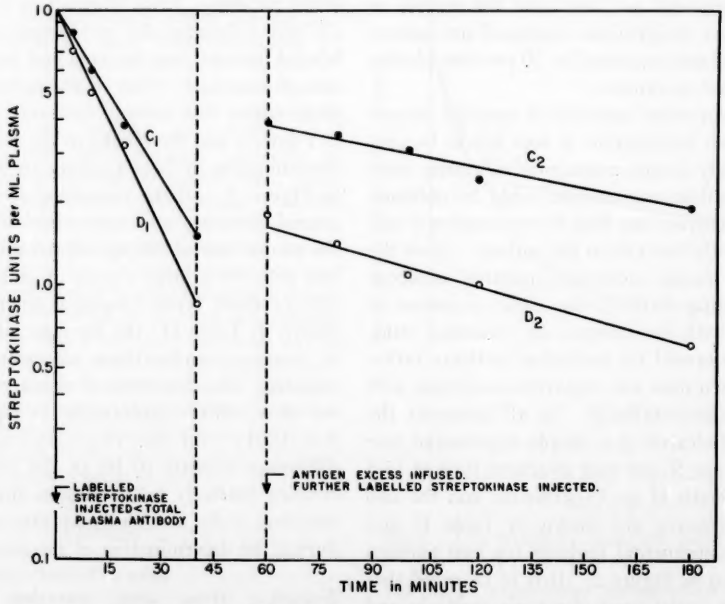

FIG. 2. THE "FAST" AND "SLOW" STREPTOKINASE PLASMA CLEARANCE RATES IN TWO PATIENTS

The patients cleared the injected streptokinase at the "fast" rate from 0 to 40 minutes and at the "slow" rate from 60 minutes onward.

fibrinolytic state with 35,000 units per hour, patients with an initial antibody of 50,000 to 100,000 units require 50,000 units per hour, whereas those with higher initial plasma antibody totals usually need 100,000 units per hour. Obviously, under these experimental circumstances, the actual initial plasma antibody content has no direct relevance to the mechanism of antigen clearance and is being used solely as a measure of the patient's immunological status towards streptokinase. Fifteen of these patients received infusions lasting 30 to 40 hours and in all there was clear biochemical evidence that the clearance rate of streptokinase remained at least relatively constant throughout the infusion period. The fibrinolytic activities assayed were similar to those recorded with labeled streptokinase, slowing of the infusion rates caused a reduction in the biochemical activities and within two to three hours of stopping the infusion all fibrinolytic activity disappeared. Since the apparent space distribution of human albumin at equilibrium has been found to be 2.5 times the plasma volume (14), these long infusion periods exclude intravascular-extravascular equilibration as being the cause of the present finding.

Breakdown of the antigen and organ localization

Catabolism of the labeled material was rapid, 22 to 55 per cent of the injected I^{131} being excreted in the urine in 24 hours, when doses of 4 to 15 microcuries were injected. All the urinary I^{131} was in dialysable form and at the end of this period a substantial thyroid uptake of I^{131} was demonstrable; this I^{131} was assumed to have been taken up by the thyroid after degradation of the protein molecule. Unbound I^{131} could be detected in the plasma shortly after the injection of the labeled material and the proportion of unbound to protein bound I^{131} showed an increase with time; usually the free I^{131} was 3 to 6 per cent of the total during the first 45 minutes. Later, however, the ratio of free to protein bound I^{131} increased; by 6 hours free I^{131} was 20 to 30 per cent of the total and by 24 hours, 40 to 50 per cent of the total. The significance of these later readings is somewhat uncertain as the protein bound component may not represent labeled streptokinase.

External monitoring with a directional scintillation detector was performed in two patients. The somewhat higher counts recorded over the liver and spleen were suggestive rather than conclusive evidence of antigen localization within these organs.

Change in the quantitative ability of the human to clear injected antigen at the fast rate following antigenic stimulation

Since the "fast" clearance rate involves the combination of antigen and antibody, the increase of circulating antibody following specific antigenic stimulation should increase the ability of man to clear injected antigen at the "fast" rate. This effect was demonstrated in two patients who received streptokinase therapy and as a consequence became immunized.

The first patient was shown on the basis of *in vitro* testing to require 30,000 units of streptokinase to induce fibrinolysis; he received 50,000 units intravenously and fibrinolysis was produced. Immediately following the onset of fibrinolysis, labeled streptokinase was injected and the 50 per cent clearance rate was shown to be 92 minutes; that is, the material was cleared at the "slow" rate. The patient then received 1.2 million units of streptokinase over 3 days, and 10 days later he had become immunized to the extent that *in vitro* testing indicated that he would require a dose of 300,000 units to induce fibrinolysis. He received 200,000 units intravenously and his blood failed to exhibit the phenomenon of fibrinolysis; an immediate clearance with labeled material showed that streptokinase was being cleared at the fast rate (50 per cent clearance in 14 minutes). A second patient, who received 2.4 million streptokinase units over three days and became immunized, was tested in a like manner and essentially similar results were recorded. Thus the high plasma antibody level following immunization had resulted in a markedly increased ability to clear streptokinase at the "fast" rate. A patient who had received streptokinase, but who had not become immunized, showed no increased ability to clear injected antigen by the "fast" rate.

Clinical effects of the antigen injections

The usual expectation following the intravenous injection of antigen into immunized man or animals is that a degree of clinical upset will occur

amounting in the severer cases to anaphylactic shock or even death. Not infrequently a subsequent episode of "serum sickness" may result.

It has been recently reported that immune man tolerates without substantial clinical disturbance the infusion of streptokinase in doses of 2 to 5 micrograms antigen N per Kg. body weight over a four hour period (7). However in the present work doses of up to 10 micrograms antigen N per Kg. body weight have been injected over a 10 minute period without detectable clinical disturbance in immune man. Furthermore, following the use of initial large doses to remove circulating antibody, maintenance of the desired intense fibrinolytic state (5) has required the infusion of 0.75 to 2.5 micrograms antigen N per Kg. body weight per hour for periods of 30 or more hours. Deleterious clinical effects attributable to the antigen injection have been absent and serum sickness has not subsequently developed though on occasion, mild hemorrhagic phenomena related to the intensity and duration of the fibrinolytic state have been noted (5). Thus man appears tolerant of this antigen (at least within the stated dose limits) by intravenous injection during states of antibody excess, antigen-antibody equivalence and vast antigen excess.

DISCUSSION

It is apparent that in man, as in the animal, there exist two rates of plasma clearance for antigenic proteins from the blood stream. If specific antibody is involved the antigen is cleared at a relatively great rate, but if specific antibody is absent a much slower rate of clearance occurs.

The following reasons are held to justify the reference in the text to the streptokinase inhibitory material as antibody. *1*) The inhibitory material is present in the gamma globulin fraction. *2*) Streptokinase administration causes, after a latent period, a great rise in the serum gamma globulin capacity for streptokinase inhibition. *3*) The length of the latent period is variable, but consistent with that found with other antibody systems and on the whole is shorter when the initial level of inhibitory material is high (booster effect). *4*) Following the latent period, the rise of inhibition is sustained for many months. *5*) The concentrations of serum inhibitory material are distributed in the population as a log-normal variable—the common type of distribution found for antibody levels.

The clearance results obtained were not attributable to the denaturation of the labeled streptokinase by the iodination process, as each batch of labeled material was shown to be cleared at either the fast or slow rate, under appropriate circumstances. No batch of labeled material used in man suffered loss of specific biochemical activity and the biological testing showed that each individual batch of material yielded essentially similar results. However extrapolation of the plasma clearance lines to determine the theoretical plasma concentration at zero time and using a plasma volume calculated from the patient's weight showed that frequently the estimated zero time plasma concentration of labeled material was only 80 per cent of the calculated theoretical concentration. This suggested that a minor portion of the injected antigen could have been cleared in a manner different from that of the major portion. This hypothesis receives support from the work of McFarlane (15), who compared the clearances of C^{14} (biologically labeled) and I^{131} trace-labeled proteins in rabbits and found that sometimes a portion of the I^{131} trace-labeled protein was removed in a manner different to that of the C^{14}-labeled proteins. He showed that this discrepancy could be corrected by "filtering" the I^{131}-labeled protein in the circulation of an animal. However it is unlikely that serious error was introduced by this failure to account for all the injected material as the discrepancy was comparatively small and the anomaly was only evident in the moments required for equilibrium prior to the withdrawal of the first sample.

It is improbable that stripping of the protein label, apart from true protein catabolism, played any significant role in the results, though Freidberg, Walter and Haurowitz (16) have shown that this may be a serious source of error in determining concentrations of tissue antigens after a period of days. First, clearance rates were measured for only two to three hours; secondly, only plasma clearance rates rather than tissue concentrations were measured; and thirdly, control experiments showed that the label remained protein bound when the labeled streptokinase was incubated with plasma.

No method is at present available for the direct determination of streptokinase in plasma and as a consequence quantitation of plasma streptokinase rested solely upon assay for the radioactive label. However, indirect biochemical evidence, evinced through measurement of the components of the plasminogen-plasmin system, has been consistent with the hypothesis that the label and biochemical activity remained associated under *in vivo* conditions. Though it has been shown in man that I^{131} trace-labeled human albumin, prepared by a variety of techniques, may be cleared at different rates from the body (14), these differences were only detectable over a period of days and the present experiments were conducted over a period of minutes. Moreover there is *in vitro* evidence that labeling with I^{131} may leave the immunological properties of an antigen unchanged (9) and *in vivo* evidence that the ratio of label to immunological activity of the protein remains constant for a period of days (17, 18). Lastly there is recent evidence in the rabbit that the anomalies in I^{131} label clearances previously reported were a function of the technique used rather than of the method itself (15, 19).

The "fast" rate of plasma clearance appears to result as a consequence of antigen-antibody union and the removal of this complex. It is of peculiar interest that, under these experimental circumstances, the antigen-antibody complex remains in the soluble form without precipitation, in fact the clearance mechanism is of such an effective nature that it is difficult to conceive of a situation in which a precipitating antigen-antibody complex could be formed. This situation appears to be wholly different from that obtaining in the rabbit where the occurrence of the "fast" clearance is apparently dependent upon the formation of a precipitating complex (3). Since, under these circumstances in man, the concentration of antigen and antibody does not need to approach the precipitating concentrations in order for the fast clearance rate to occur, it would seem likely that this fast clearance of antigen may be found to occur universally in immunized man and may thus be a biological phenomenon of the widest significance.

The "slow" rate of plasma clearance had an average half time of 83 minutes and showed a wide variation. None of the material appears as antigen in the urine. Breakdown rates, as far as they could be judged by determining the ratios of free and protein bound I^{131} in the plasma and the excretion of this material in the urine, appeared to be equally high for material cleared by either the "fast" or the "slow" rate. Though these rates may have been influenced by the presence of extravascular antibody, the high antigen/antibody ratios used in some experiments exclude this factor as a major source of error.

Most of the patients in whom clearance studies were performed suffered from thrombophlebitis and, theoretically at least, the clearance results could have been influenced by the absorption of streptokinase upon thrombi. However *in vitro* measurement of the uptake rates of labeled streptokinase upon human plasma clots reveal that in practice this factor probably exerted no influence upon our experimental findings. For instance labeled streptokinase was absorbed onto large plasma clots (approximate volume 400 cu. mm.) at the rate of 3.1 ± 1.1 streptokinase units per clot per 30 minutes. These determinations were made using plasma containing 15 free streptokinase units per ml. (higher concentrations than were ever attained *in vivo*) and indicate a negligible absorption compared to the large doses infused.

The results of the long term infusions with both labeled and unlabeled streptokinase appear to be of considerable significance. Since both labeled and unlabeled materials were treated similarly the influence of labeling artifact can be eliminated. Though precise estimates of plasma clearance rate could not be made, the results provide conclusive evidence that, at least within a single order of magnitude, the plasma clearance rates remained relatively stable throughout the duration of infusions lasting as long as 30 hours. This conclusion has important implications with regard to the clearance mechanisms since the 50 per cent clearance rate of a nonantigenic protein of comparable molecular weight, human albumin, has been variously determined to be 10.5 to 26.5 days (20, 21).

On both a temporal and a quantitative basis it seems necessary to postulate increased activity of the reticulo-endothelial system to explain this great difference in plasma clearance rates between the antigenic and the nonantigenic protein. Unfortunately, though all our patients showed comparable clearance rates, streptococcal infection is

so common an event during life that it is not possible to decide whether the increased ability to clear streptokinase represents an innate or an immunologically acquired quality. However suggestive evidence that this clearance mechanism may be immunologically determined comes from the observation that the streptokinase infusion rate required for the maintenance of a steady fibrinolytic state can be predicted from the initial antibody level.

These experiments suffer from the defect that the clearance of only one highly purified antigen has been studied. Other antigens might be treated in a different manner, though the general identity of these reactions with those reported to occur in animals would suggest that a phenomenon of general rather than of specialized interest has been studied.

SUMMARY

1. The plasma clearance rates of streptokinase, a protein antigenic to man, have been studied in both normal and immune man. Preparations of streptokinase, biophysically homogeneous, were trace labeled with I^{131} and were used to determine plasma streptokinase levels.

2. Intravenously injected streptokinase was cleared from the circulation at two distinct rates, the "fast" (average 50 per cent plasma clearance time 18 minutes), and the "slow" (average 50 per cent clearance time 83 minutes). The fast rate was a consequence of the combination of the antigen with its antibody, and the clearance of the complex. The "slow" rate was shown to be operative in the absence of plasma antibody and may be due to activity of the reticulo-endothelial system.

3. Specific immunization of the patient was shown to result in an increase in the proportion of antigen cleared at the fast rate. Doses of up to 10 micrograms antigen N per Kg. body weight were used in immune man, and the lack of deleterious clinical response was thought to be due to the patient's acquired ability to remove, sequestrate and destroy the antigen.

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APPENDIX

Assay for streptokinase antibody

The assay for streptokinase antibody is complicated by the presence of both plasminogen and antiplasmin in serum. Since the antigen-antibody complex is of the nonprecipitating variety, the assay must depend upon the inhibitory effect of serum, or the immunologically controlled components of serum, upon streptokinase. The use of untreated serum for this assay is inexpedient as before antigen-antibody union has occurred; plasminogen may be activated by streptokinase and later the presence of antiplasmin will interfere with the use of a biochemical indicator system.

The customary technique used for this assay, that of Christensen (11), was found to yield erroneously high values, since the period of heating at 56°C., recommended for the purpose of denaturing serum plasminogen and serum plasmin inhibitor, was unsatisfactory for this purpose. Though plasminogen became denatured in a biochemical sense, the denatured material bound streptokinase and thus the Christensen assay measured both this property and the true antibody content. Altered experimental conditions failed to obviate this anomaly and a satisfactory correction could not be applied as serum plasminogen concentration is not a fixed quantity. Accordingly streptokinase antibody assays were performed upon the isolated gamma globulin fractions of serum prepared by the technique of Lever and co-workers (12) modified only insofar as the precipitates were separated by centrifugation rather than by sintered glass filtration. This technique permitted a virtually quantitative extraction of electrophoretically homogeneous gamma globulin from the serum, which was also plasminogen free.

The success of each fractionation was judged by performing paper electrophoretic separations to demonstrate homogeneity and testing by optical density readings at 280 mμ that 9 to 13 per cent of the original serum protein was present in the isolated fraction. Some 10 per cent of samples had to be refractionated so as to comply with these criteria.

The antibody content of the serum gamma globulin fraction was estimated by a modified Christensen method (11). *1)* To each of seven tubes containing 0.4 ml. gamma globulin solution was added, by means of a microburette[4] (containing streptokinase 500 units per ml.), 1 to 50 streptokinase units; *2)* the tubes were incubated at 37°C. for 30 minutes; *3)* 0.2 ml. of a 0.25 per cent solution of Fraction III,[5] 0.2 ml. of 0.5 per cent fibrino-

[4] Micrometric Instruments, Cleveland, Ohio.

[5] Cohn Fraction III prepared from human plasma. Obtained through the courtesy of the American National Red Cross from E. R. Squibb and Sons.

gen [6] and 0.1 ml. of thrombin [7] (*2* units) were added to each tube; *4*) the tubes were incubated at 37°C. and observed for lysis over a two hour period; *5*) the reciprocals of the lysis times for periods exceeding 20 minutes were plotted against the log streptokinase concentration; *6*) the resulting straight line plot was extrapolated to infinite time and the quantity of streptokinase inhibited for an infinite time by the specimen was read off from the graphic intercept; *7*) the original antibody content of the specimen was calculated and the result expressed as streptokinase units inhibited per ml. of serum.

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[6] Bovine fibrinogen, 92 per cent of the nitrogen being clottable by thrombin. Kindly supplied by Dr. Kent Miller of the New York State Laboratories, New York.

[7] Parke Davis bovine thrombin.

A STUDY OF THE EFFECTS OF EXCESSIVE POTASSIUM INTAKE UPON BODY POTASSIUM STORES[1]

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There is extensive evidence that provision of potassium as well as sodium may be of vital importance in the treatment of a wide variety of metabolically disturbed patients (1–3). Physicians sometimes are hesitant to administer potassium, however, because they are concerned that such therapy may result in potassium intoxication. The present studies were undertaken in an effort to gather information which would facilitate considerations of this problem.

Specifically, the aims of this study were threefold: The first was to determine the upper limit of potassium intake compatible with body potassium homeostasis. The second was to determine the nature of the variables which the body's homeostatic systems are attempting to protect under conditions of potassium loading. The third was to find out to what extent, if any, the chronically loaded individual can store surplus potassium in innocuous form when intake exceeds excretory capacity. Because these investigations of necessity involved the administration of potentially lethal doses of potassium, they have been carried out on rats rather than human beings.

MATERIALS AND METHODS

All experiments were performed on healthy male Sprague-Dawley rats weighing between 150 and 200 Gm. The animals were individually caged and provided with tap water *ad lib*. Control animals received approximately 7 Gm. of Purina rat chow per 100 Gm. of body weight per day. This provided the animal with approximately 1.6 mEq. of potassium and 0.8 mEq. of sodium per 100 Gm. of body weight per day. Potassium loaded rats were allowed 6 to 7 Gm. of the same feed in powdered form to which was added a solution containing 3.54 mEq. of potassium, 2.66 mEq. chloride and 0.88 mEq. bicarbonate per ml. This mixture was used in an effort to avoid deviations in extracellular pH which may of themselves cause shifts of potassium within the body (4). Each animal was caged separately and due attention was given to physical cleanliness. Acceptance of feed was estimated within 1 Gm. daily and potassium dosages were adjusted for changes in rat size at five day intervals. All animals were anaesthetized with intraperitoneal pentobarbital sodium prior to sacrifice. Electrocardiograms were obtained during light anaesthesia (30 mg. per Kg. body weight) with a Sanborn Twin-Beam photographic apparatus at a paper speed of 75 mm. per second and internal calibration of 1 cm. equal to 1 mV. The tracings were measured by caliper. Blood specimens for determination of plasma pH (5), carbon dioxide content (6), sodium (7), potassium (7) and chloride (8) were collected from the vena cava in heparinized syringes and delivered beneath oil for centrifugation. Immediately thereafter a representative sample of the vigorously contracting cardiac ventricles was excised, blotted free of blood and subjected to separate analysis. The intestinal tract from the esophago-gastric junction to the rectum was then removed and discarded. The heart muscle sample and the remainder of the carcass were weighed, dried to constant weight, defatted in equal parts of ethyl and petroleum ethers, dried again to constant weight and extracted with 0.75 N nitric acid for determination of sodium (7), potassium (7) and chloride (9). Cardiac chloride was determined polarographically (8) on an 0.75 N nitric acid extract of 0.3 to 0.5 Gm. of dry, defatted tissue made up to a volume of 10 ml. prior to filtering and analysis.[2]

Data for the extracellular and intracellular volumes and electrolyte distribution were calculated assuming that all chloride is extracellular and present at a concentration equal to that of an ultrafiltrate of serum.[3] In

[1] This work was supported by Grants A-808 of the National Institute of Arthritis and Metabolic Disease, by Grants H-1529 and HTS-5139 from the National Heart Institute, Public Health Service and by a grant from the Commonwealth Fund of New York.

[2] The titration and polarographic methods for chloride were compared by application to eight samples of filtered, nitric acid carcass extract in which chloride concentration ranged between 13 and 17 mEq. per liter and to eight samples of serum in which the concentration of chloride ranged between 96 and 114 mEq. per liter. Analysis of the variance yielded a standard error of 0.358, a t value of 0.183 and a p value of 0.5.

[3] Ultrafiltrate concentration values were derived from serum concentration values by application of an assumed constant serum water value of 0.93 and a Donnan equilibrium factor of 0.95 (10).

calculating intracellular potassium concentrations, correction was made for the small extracellular potassium content of the tissues. The control electrocardiograms and serum and heart analyses carried out during the present study were supplemented by carcass analyses of normal rats of the same strain, sex, weight and dietary intake performed in this laboratory in the course of a previous study (11). In group comparisons, differences have been considered significant where the value for p is equal to or less than 0.05 using the t test.

OBSERVATIONS

Figure 1 demonstrates the relationship of the animals' potassium intake (abscissa) to the concentration of potassium in the extracellular and to the concentration and content of potassium in the total carcass and cardiac intracellular compartments. The individual control data are shown at the ordinary intake level of 1.6 mEq. of potassium per 100 Gm. body weight per day and the normal 95 per cent distribution as found for 25 animals (1.97 standard deviations) is represented by the horizontal shaded bars.

When the potassium intake was altered by 8 mEq. per 100 Gm. body weight per day stepwise increments at five day intervals, the animals refused small portions of feed on the first day of each new intake level, but otherwise remained apparently well at intakes of 9.6, 17.6 and 25.6 mEq. per 100 Gm. body weight per day. Marked diuresis and polydipsia were noted with the increasing osmotic load, but no other changes in appearance or activity were observed. A seven per cent mean weight gain occurred in these experimental animals during the course of the 15 days. Plasma potassium concentration determined in four or five animals which were sacrificed for this purpose at the end of each five day period remained within the normal range as noted in the top section of Figure 1.

When rats were subjected to the next higher dose, *i.e.*, 33.6 mEq. per 100 Gm. body weight per day, continuing partial refusal of the potassium-containing feed occurred with the result that the net potassium intake of certain rats was less than the dose offered. The animals were therefore maintained on this feeding for ten days, and were then offered a diet containing 38.4 mEq. per 100 Gm. body weight per day in an effort to attain the 33.6 mEq. per 100 Gm. body weight per day net intake level in all animals. By the 14th day of potassium loading in excess of 25.6 mEq. per 100 Gm. body weight per day, half of the 16 animals had died, each within three days of attaining its individual maximum intake (27.5 to 37.9 mEq. per 100 Gm. per day or about 60 mEq. per rat per day). The experiment was terminated at this predetermined end-point of 50 per cent deaths (LD_{50}) by the sacrifice of the eight remaining animals. These sacrificed animals had lost weight terminally to a mean of 92 per cent of initial weight and evidenced the same symptoms noted in the animals that died, namely, a progressive tendency toward roughening of coat, irritability, lethargy and eventually generalized weakness. The maximum potassium intakes in the sacrificed animals (29.4 to 37.9 mEq. per 100 Gm. per day) had also been attained between one and three days prior to sacrifice. At sacrifice, these animals had gross evidence of adrenal enlargement.

Table I shows that the plasma potassium and chloride concentration values of the LD_{50} survivors were significantly higher than those of animals on the 1.6 mEq. per 100 Gm. body weight

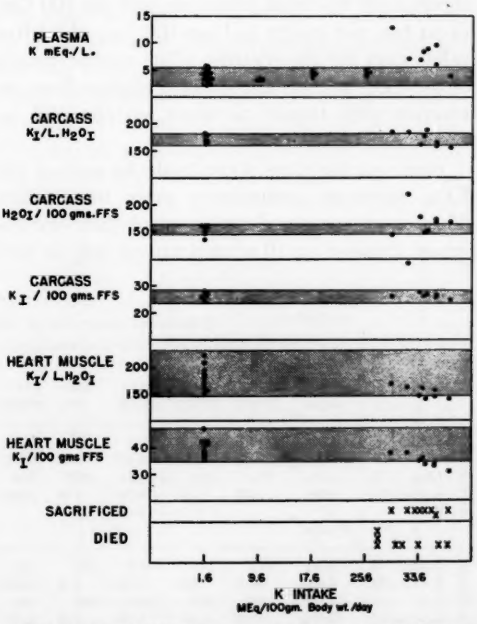

FIG. 1. RELATIONS BETWEEN K INTAKE AND EXTRACELLULAR AND INTRACELLULAR CONCENTRATION AND CONTENT VALUES

I stands for intracellular, FFS for fat free solids.

TABLE I

*Plasma concentrations**

		pH	CO_2	Cl	K	Na
Controls	M	7.43	26.7	101	4.15	143
	SE	0.0092	0.679	0.753	0.147	0.472
	N	8	8	19	25	19
LD_{50} survivors	M	7.42	27.5	108	8.0	143
	SE	0.0187	0.522	1.875	0.984	2.067
	N	8	6	8	8	8
p				0.01	0.01	

* CO_2 content, chloride, potassium and sodium are expressed as mEq. per liter of plasma. M = mean; SE = standard error of mean; N = number of animals; *p* = significance level. Plasma CO_2 was not determined in two LD_{50} survivors with plasma pH of 7.45.

per day potassium intake. However, as can be seen in the upper half of Table II, there was no significant difference between the LD_{50} survivors and the controls with respect to the total potassium content or to the concentration of potassium in cell water of the carcass as a whole.

The lower half of Table II shows that the cardiac muscle of the LD_{50} survivors contained significantly less total potassium both per 100 Gm. of fat free wet weight and per 100 Gm. of fat free solids than did the controls. The cardiac muscle of the LD_{50} animals also differed slightly from the controls with respect to water, sodium and fat content.

Electrocardiograms were made in each of the LD_{50} survivors immediately prior to sacrifice. These were compared with control electrocardiograms obtained on 10 normal rats as well as with a series gathered on rats subjected to acute potassium intoxication by intraperitoneal loading. The acute loading had resulted sequentially in bradycardia, P wave voltage depression, T wave voltage *depression* or *inversion,* PR and QT interval prolongation, QRS widening and, terminally, sine wave formation; Table III shows that the chronically loaded LD_{50} animals also showed significant changes in the first three of these variables. As can be seen in Figure 2, the changes in T wave voltage bore an inverse relation to plasma potassium concentration; there also was a statistically significant correlation between T wave voltage and potassium concentration per liter of cardiac intracellular water.

COMMENTS

In defining the ceiling, or upper limit of body tolerance for a substance, two levels may be considered: *a*) an upper lethal limit, and *b*) a lower physiologic ceiling at which pathologic alteration of body composition or concentration first occurs. It might be anticipated that the location of the intoxication zone defined by these parameters would differ among individuals as a result of biologic variability. Thus it appears from Figure 1 that the upper lethal limit for rats under the circumstances of the present experiments is between 27.2 and some quantity in excess of 38.4 mEq. per 100 Gm. of body weight per day. The fact that all but one of the LD_{50} survivors showed both hyperkalemia and characteristic electrocardiographic changes indicates that these individuals were ingesting po-

TABLE II

Potassium concentration in total carcass and cardiac muscle

			Fat % total weight	H_2O_T FFWW	H_2O_E FFWW	H_2O_I FFWW	H_2O_I FFS	Cl_T FFWW	K_T FFWW	K_E FFWW	K_I FFS	K_I LICW	Na_T FFWW	Na_E FFWW	Na_L FFS	Na_I LICW
Total Carcass	Control	M	2.62	72.1	29.2	42.6	154	3.28	7.46	0.13	26.2	175	5.02	4.24	2.78	18.1
	N=8	SE	0.277	0.322	0.336	0.456	3.32	0.0445	0.0883	0.0068	0.430	1.91	0.0671	0.0421	0.225	1.4
	LD_{50}	M	1.11	73.1	29.9	43.2	162.8	3.52	7.67	0.23	28.0	172	5.29	4.36	3.53	21.5
	survivors	SE	0.325	0.859	0.742	0.714	7.88	0.0940	0.194	0.0270	1.56	4.60	0.1059	0.0750	0.369	1.88
	N=8															
		p	<0.01													
Cardiac Muscle	Control	M	0.092	78.1	28.7	49.4	226	3.30	8.88	0.11	40.6	178.4	4.63	4.19	2.14	9.20
	N=11	SE	0.0184	0.200	0.650	0.702	4.21	0.0760	0.226	0.0078	1.044	7.20	0.1189	0.0800	0.602	2.39
	LD_{50}	M	0.288	77.2	25.2	52.1	229	2.99	8.10	0.20	35.6	152	3.90	3.56	1.49	6.38
	survivors	SE	0.0781	0.164	0.698	0.669	3.35	0.1017	0.166	0.0287	0.828	3.90	0.1321	0.0731	0.442	2.61
	N=8															
		p	<0.05	<0.05		<0.05			<0.05		<0.01	<0.025	<0.01	<0.005		

* Values for chloride, potassium and sodium (MEq.) and water (Gm.) are expressed per 100 Gm. of fat free wet weight (FFWW), per 100 Gm. fat free solids (FFS) and per liter of intracellular water (LICW). Subscript T = total; E = extracellular; I = intracellular.

TABLE III

Electrocardiographic standard in 10 normal male Sprague-Dawley rats weighing 150 to 200 Gm. and in the present LD_{50} survivors during light pentobarbital anaesthesia

	Controls		LD_{50} survivors		
	Mean	SD	Mean	SD	p
Ventricular rate per min.	390	14	321.9	+37.0	<0.001
P voltage, lead II, mV.	0.16	0.03	0.095	+ 0.04	<0.01
T voltage, lead II, mV.	+0.16	0.03	+0.08	+ 0.07	<0.025
PR interval, msec.	46.5	2.8	48.0	+15.0	>0.5
QT interval, msec.	56.4	3.6	51.5	+18.3	>0.5
QRS, msec.	13.8	0.7	16.8	+ 6.9	<0.4

tassium at rates which were in excess of their respective physiologic ceiling levels. The absence of diarrhea in these animals suggests that the measured intake ceilings were, in fact, indirect estimations of renal excretory capacity for potassium ion. In confirmation of this, one animal accepting 32 mEq. per 100 Gm. body weight per day was found to be excreting 97.5 per cent of this potassium in the urine.

The LD_{50} survivors appeared to be limiting their intake somewhat in accordance with their degree of hyperkalemia (*i.e.*, intoxication). Though this reaction might be considered protective in nature, it failed to prevent the spontaneous ingestion of lethal doses of potassium. Inasmuch as this phenomenon has been observed also in patients with renal failure, it appears that one cannot depend upon instinct to protect human beings against taking a lethal load of potassium by mouth in the form of fruit juice or other ordinary nutrient. Fortunately, it is possible to estimate an individual's tolerance and to prescribe accordingly (12).

The data obtained on animals taking between 1.6 and 27.2 mEq. of potassium per 100 Gm. body weight per 24 hours reveal that extracellular concentration values are strongly defended over a wide intake range. The close similarity in the carcass and cardiac values obtained on the control and LD_{50} animals indicates that the same is true also with respect to intracellular content and concentration values. It may be inferred therefore that the body's potassium homeostatic system is designed to prevent appreciable deviations in the potassium status of either the extracellular or the intracellular compartment. On the other hand, the fact that animals chronically overloaded with potassium develop electrocardiographic and other evidences of potassium poisoning in association with elevated extracellular, but normal total carcass and slightly lowered cardiac intracellular concentration and content values suggests that potassium intoxication as it occurs in normal individuals is largely an extracellular phenomenon, a conclusion reached by others on the basis of plasma and isolated muscle tissue measurements (13).

It is conceivable that these rats might have shown more of an increase in cellular potassium content had their caloric intake and hence glycogen stores been sustained by forced feeding immediately prior to death (14). Even so, however, it can be appreciated that such retention could afford only extremely limited protection under conditions of prolonged potassium loading. Moreover, the behaviour of these animals was quite characteristic of potassium intoxicated individuals who are apt to be weak and anorexic. It follows

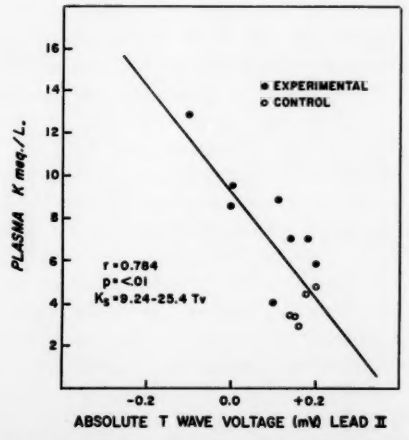

FIG. 2. RELATIONS BETWEEN ELECTROCARDIOGRAPHIC T WAVE VOLTAGE AND THE CONCENTRATION OF POTASSIUM IN PLASMA

	AUTHORS		ANIMAL	EXTRACELL. pH	DATA	COMMENT
ACUTE K LOAD	◊ BOURDILLON	(15)	MAN	NOT REPORTED	EXTERNAL BALANCE	EXP. 6 + 7
	⊠ WINKLER + SMITH	(16)	DOG	NOT REPORTED	EXTERNAL BALANCE	REPORTED Δ K'S
	▼ SCRIBNER ET AL.	(17)	DOG	NORMAL	EXTERNAL BALANCE	CONTROL GROUP
	● MILLER - DARROW	(13)	RAT	NOT REPORTED	MUSCLE ANALYSIS	GROUPS 6, 7 + 8
	X PRESENT AUTHORS		RAT	NORMAL	EXTERNAL BALANCE	LIGATED URETERS
ADRENALECTOMY	□ DARROW ET AL	(18)	CAT	NOT REPORTED	"	MARKED INSUFF.
	● DARROW ET AL	(18)	DOG	NOT REPORTED	"	MOD. INSUFF.
	○ HARRISON + DARROW	(19)	RAT	NOT REPORTED	"	MARKED INSUFF.
	▼ ROLF + WHITE	(20)	RAT	NOT REPORTED	"	MARKED INSUFF.
	◊ CONWAY + HINGERTY	(21)	RAT	7.14	"	MEAN DATA

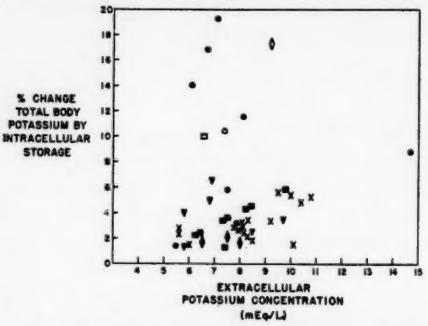

FIG. 3. PER CENT INCREASE IN CELLULAR POTASSIUM ABOVE AVERAGE NORMAL FOLLOWING ELEVATION IN EXTRACELLULAR POTASSIUM CONCENTRATION BY *ACUTE* POTASSIUM LOADING

that prevention of hyperkalemia under these circumstances depends almost exclusively upon homeostatic adjustment of renal potassium output. While this mechanism works admirably over a much wider range of intake than the normal individual is apt ever to experience, there is an upper limit to renal excretory capacity. Once this has been reached, even small increments in potassium intake in excess of maximal bodily excretory capacity can result in the production of hyperkalemia of toxic degree (12). From this interpretation, it may be concluded that the ceiling zone for potassium under conditions of chronic loading is narrow in a given individual.

The absence of intracellular potassium storage in the present chronic loading experiments is in contrast to the storage which may be observed immediately following an acute potassium load. Thus Figure 3, which presents observations on rats, dogs and man, shows that intracellular storage amounting to as much as 6 per cent of normal body potassium content may occur when the extracellular potassium concentration is abruptly elevated to 10 mEq. per liter (13, 15–22).[4] In the rat, the quantity thus stored is equal to about one quarter of the animal's usual daily intake (about 2.5 mEq. per 180 Gm. rat); in the adult human, it is equivalent to about 150 per cent of a commonplace total daily intake (about 120 mEq.). It has been found that the extra potassium stored in cells following such an acute load normally is eliminated within one to three hours (13, 25, 26). Perhaps this is fortunate, for otherwise the capacity of the cellular compartment to buffer tendencies to hyperkalemia after high potassium meals might soon become saturated.

Figure 3 also shows that the cell potassium is much higher relative to the extracellular concentration in adrenalectomized animals than in normal animals. This suggests that adrenal hormones may have a direct effect on the extracellular intracellular concentration gradient for potassium (27, 28). In view of the evidence that potassium loading is a potent stimulus to aldosterone secretion (29), the possibility arises that such adrenocortical activity may have been the factor which minimized cellular potassium storage in the body as a whole and actually caused lowering of the cardiac cellular potassium in the chronically loaded animals here reported. Because some time must elapse before the adrenals can react fully to a

[4] Data from the literature were calculated to standard units utilizing the following assumptions: *1.* Normal initial body potassium 45 mEq. per Kg. in man and appropriate normal values for dog, cat and rat tissue (23, 24). *2.* Per cent change in muscle composition reflects per cent change in total body intracellular potassium. *3.* Initial serum potassium concentration (Ks) equals 4.0 mEq. per liter when Δ concentration reported. In addition to the data shown in Figure 3 are data derived from rats identical to the control group reported in the main body of the experiment. These animals underwent ureteral ligation after a control blood sample was drawn by cardiac puncture. Within five minutes of ureter ligation, the abdomen was closed tightly and potassium was administered intraperitoneally as a 4 KCl : 1 $KHCO_3$ solution containing 0.58 mEq. potassium per ml. Dosage ranged from 2 to 6 mEq. per Kg. body weight. The animals were sacrificed at one or two hours after potassium administration by exsanguination as described. Intracellular potassium storage was calculated from the known dosages and initial and final plasma concentrations of chloride and potassium, correcting for minimal changes in weight and assuming the initial body water composition shown for control animals in Tables I and II. Plasma pH was not altered in the final blood specimen when determined in representative animals. Control animals subjected to the same procedure without the potassium load showed unaltered pH values and changes in plasma potassium concentration ranging from − 0.6 to + 0.4 mEq. per liter.

potassium load, such a mechanism would permit body cells to store potassium temporarily following an acute load, but would prevent the storage of potassium under conditions of sustained hyperkalemia. These findings also suggest that cardiac muscle may differ from other body protoplasm in its responsiveness to these adrenocortical hormones.

SUMMARY AND CONCLUSIONS

Rats subjected to loads of potassium which were increased stepwise from a normal intake level of 1.6 to as high as 36.8 mEq. per 100 Gm. body weight per day showed no signs of toxicity, electrocardiographic changes or elevation of plasma potassium concentration values until the intake exceeded 25.6 mEq. per 100 Gm. body weight per day. Of the animals taking more than this amount, one half died with signs of potassium intoxication within three days after attaining intake levels ranging between 28.8 and 38.4 mEq. per 100 Gm. body weight per day. Measurements carried out on the surviving half of the group disclosed pathologic elevation of plasma potassium concentration and characteristic electrocardiographic changes, but no significant change in total carcass potassium content or concentration values and a significant decrease in cardiac intracellular potassium concentration. These findings were in contrast to data obtained on animals on an ordinary dietary intake in which some intracellular "storage" of potassium may be observed transiently following the administration of an acute load. It is suggested that the restoration of cellular potassium values to normal levels following an acute load and the maintenance of these values at normal levels even in the presence of sustained hyperkalemia under conditions of chronic loading may be mediated by adrenocortical hormones.

It is concluded that the occurrence of sustained hyperkalemia means that the individual has surpassed the upper limit of his individual tolerance for potassium and that he probably has very little capacity to store additional potassium within the body in innocuous form.

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APPLICATION OF CONTINUOUS FLOW ELECTROPHORESIS TO THE STUDY OF THE BLOOD COAGULATION PROTEINS AND THE FIBRINOLYTIC ENZYME SYSTEM. I. NORMAL HUMAN MATERIALS [1]

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Continuous flow filter paper electrophoresis, as developed by Svensson and Brattsen (1), Grassmann and Hannig (2–4) and Durrum (5), offers a method of plasma fractionation in which protein denaturation appears minimal. This study involves the testing of such fractions for biological activity in various coagulation systems. With the exception of fibrinogen, the coagulation proteins are present in plasma in minute amounts which, at the present time, can be assayed only by their activities in specific clotting systems. Many of these coagulation factors are readily denatured; thus, rigid control with particular attention to temperature is necessary to obtain good recovery of biological activity.

Previous electrophoretic studies concerning distribution of plasma coagulation proteins have employed paper electrophoresis with elutions from cut strips. Owen and McKenzie (6) found prothrombin to coincide with α_2 globulin, proconvertin (stable conversion factor) with β globulin, proaccelerin (labile conversion factor) in a variable area between γ and β peaks, antithrombin between β and α_2, and fibrinogen remaining at the point of application. Using the same technique, Frick and Hagen (7) found Hageman factor activity in a fraction located between β and γ globulin. On the other hand, the electrophoretic mobility of purified prothrombin was described by Seegers, McClaughry, and Fahey (8) as approximately equivalent to that of α_1 globulin. The present studies show considerable agreement with these findings.

In Table I are listed the coagulation proteins whose existence seems well established today. Included in the table are the abbreviations used in the subsequent text and charts together with some of the synonyms.

METHODS

Electrophoretic technique. Principles of continuous flow electrophoresis on filter paper are well described by Block, Durrum, and Zweig (9). The two instruments [3] employed in these studies were placed in a cold room with its temperature regulated to approximately 8° C. The circulating buffer was further refrigerated, entering the instrument at 1° C. All the runs described in this communication utilized a filter paper curtain, Schleicher and Schuell 470; a barbital buffer, pH 8.6; ionic strength, 0.02; and a power supply adjusted to 50 m.a. and 720 volts. Under these conditions the thermocouple readings on the curtain during electrophoresis were 18° C. Before starting electrophoresis it was important to allow the instrument to stabilize for at least

TABLE I

Coagulation proteins

AHF	Antihemophilic Factor A—Factor VIII
PTC	Plasma thromboplastin component—Factor IX—Christmas factor—Antihemophilic Factor B
PTA	Plasma thromboplastin antecedent—Antihemophilic Factor C
HAG	Hageman factor—Antihemophilic Factor D
ProA	Proaccelerin—Factor V—labile factor—AcG
ProC	Proconvertin—stable factor A) SPCA, Factor VII B) Stuart
ProT	Prothrombin—Factor II
FIB	Fibrinogen—Factor I
AntiT	Antithrombin
H	Heparin cofactor
Glass	Glass factor
ProF	Profibrinolysin
AntiF	Antifibrinolysin

[3] Beckman/Spinco Division.

[1] This investigation was supported in part by a research grant (H-2254) from the Division of Research Grants of the National Institutes of Health, United States Public Health Service.

[2] Part of this investigation was carried out during the tenure of a postdoctorate fellowship, Life Insurance Medical Research Fund.

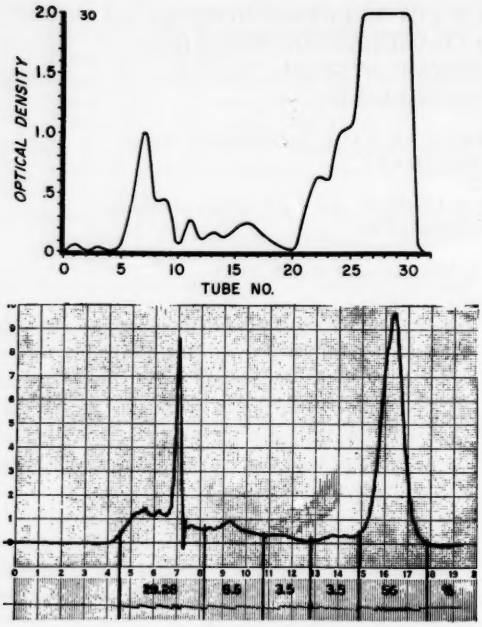

FIG. 1. COMPARISON OF CURTAIN AND PAPER STRIP ELECTROPHORESIS

six hours with buffer circulating and current applied. Plasma or its fractions were rapidly prepared, utilizing refrigerated centrifugation and dialyzing against the barbital buffer for only two hours. The feed rate was adjusted so that approximately 15 ml. of material was fractionated during the electrophoretic runs, which were usually limited to 18 hours. Depending on the rate of buffer flow down the curtain, the material separated and collected in each of 32 tubes was diluted with 5 to 10 ml. of buffer.

Ninhydrin, 0.5 ml., was added to 0.5 ml. aliquots from each tube. The tubes containing this mixture were plugged with cotton and heated for 20 minutes in a boiling water bath. Following cooling, 4 ml. of 50 per cent propanol was added and the optical densities were read at 540 mμ in a spectrophotometer. Plotting the optical density of each tube on the ordinate, with tube numbers on the abscissa, gave a rather quick semiquantitative estimate of the concentration and distribution of the protein in the tubes. Figure 1 shows that the ninhydrin optical density of each tube plotted as a continuous curve closely resembles the analytrol scan from the paper electrophoresis of the same plasma.

Although the runs were limited to 18 hours, reproducibility during a single run was determined by comparing the ninhydrin optical density of samples at 16 and 42 hour intervals (Figure 2).

Following completion of the run, the curtains were removed, dried in an oven (110° C.) and dyed with bromphenol blue. Distribution of the proteins could be further evaluated by the dye pattern.

Paper strip electrophoresis technique was the hanging strip method of Durrum (9). The experimental conditions were Schleicher and Schuell 2043 A mgl filter paper, eight strips per cell 3.0×30.6 cm., 0.02 ml. of material applied at apex, barbital buffer pH 8.6, $\Gamma/2$ 0.05, 16 hour duration, constant current of 4 m.a., and staining with bromphenol blue. The strips were analyzed by analytrol with neutral density filter calibrated 7.4 cm.

Assay of coagulation factors. With the exception of fibrinogen, the coagulation proteins were assayed in systems which attempted to be as specific as possible for the factor in question. For example, antihemophilic factor (AHF) was assayed by measuring the ability of a given fraction (or plasma) to shorten the recalcification time and improve the prothrombin consumption of a standard plasma (stored, frozen, at $-20°$ C.) from a severe hemophiliac. Obviously the recalcification time can be shortened by materials other than AHF (thrombin, thromboplastin, SPCA (10), certain cellulose products, powdered glass, and so forth). Effects of such nonspecific coagulants could not be evaluated completely, but appeared to be minor in these experiments.

The clotting activity entitled "proconvertin" in this report includes the activities of two chemically related factors: SPCA or Factor VII (11, 12) and Stuart factor (13).

"Glass factor" is a term used to express the ability of certain fractions or of plasma treated with glass to shorten the recalcification time and improve prothrombin consumption of normal plasma prepared and recalcified in siliconed tubes.

Tubes containing fractions were kept thoroughly chilled and tests carried out as rapidly as possible. The dialyzed

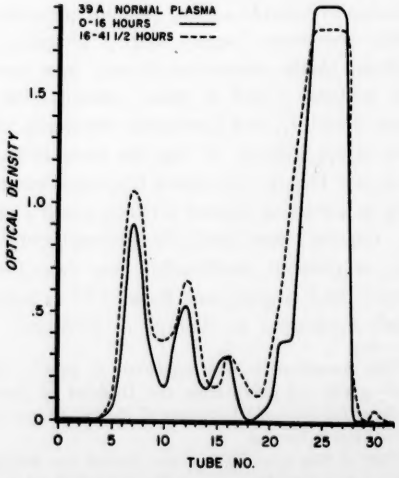

FIG. 2. COMPARISON OF 16 AND 41½ HOUR NINHYDRIN OPTICAL DENSITY CURVES

plasma remaining in the reservoir was used as the control and arbitrarily defined as containing 100 units of each factor tested. Attempts to quantitate the recovery of each factor were only partially successful as exact measurement of volume of plasma fractionated (*i.e.*, volume applied minus volume remaining on curtain) was difficult. In addition, assays such as AHF, plasma thromboplastin component (PTC), and Hageman factor have an error of approximately plus or minus 20 per cent in the range of 50 to 150 per cent of normal and plus or minus 10 per cent in the range of 1 to 50 per cent of normal. Thus most of the charts present the recovery of each factor in a semiquantitative fashion. The blocked-in areas indicate tubes containing activity comparable to from 2.5 to 100 per cent of that found in the mother plasma. When serum or chemical fractions were studied, normal plasma was used as the control.

Most of the assay methods have been previously described (14): *fibrinogen; prothrombin* (Method 1); *proconvertin; proaccelerin* (Method 1); *antihemophilic factor* (AHF); *plasma thromboplastin component* (PTC); and *Hageman factor*. In addition the following tests were included:

1. "Glass factor" assay. In siliconed tubes, mix 0.05 ml. fraction with 0.2 ml. citrated normal plasma prepared by using all siliconed glassware with centrifugation of blood at 2000 rpm for 10 minutes. Measure clotting time after addition of 0.2 ml. 0.025 M calcium chloride. After one hour at 37° C. add 0.05 ml. 0.1 M sodium oxalate, wind out the clot and measure residual prothrombin. The presence of a "glass factor," *i.e.*, a material capable of hastening coagulation of normal plasma in silicone, was evidenced by marked shortening of clotting time and lessening of residual prothrombin.

2. PTA assay (plasma thromboplastin antecedent). Sufficient material was available for assay of PTA in only one run. The system used was identical to that used for AHF, PTC, Hageman and glass factors: 0.2 ml. of deficient plasma[4] is mixed with 0.05 ml. fraction or plasma dilution, and recalcified with 0.2 ml. of 0.025 M $CaCl_2$. The clotting time (37° C.) is recorded and after one hour 0.05 ml. of 0.1 M sodium oxalate is added, the clot wound out and the residual prothrombin determined.

3. Antithrombin time. Mix 0.1 ml. fraction plus 0.1 ml. 0.85 per cent sodium chloride plus 0.1 ml. human thrombin; incubate mixture 30 minutes at 37° C.; remove clot, if any; determine clotting time on addition of 0.1 ml. bovine fibrinogen.

4. Heparin cofactor time. Mix 0.1 ml. fraction plus 0.1 ml. heparin (5 units per ml.) plus 0.1 ml. fibrinogen; determine clotting on addition of 0.1 ml. human thrombin.

5. Control for 3 and 4. Mix 0.1 ml. fraction plus 0.1 ml. 0.85 per cent sodium chloride plus 0.1 ml. fibrinogen; determine clotting on addition of 0.1 ml. human thrombin.

Preparation of human thrombin. Suspend 0.1 ml. washed human platelets (source of proaccelerin) plus 0.1 ml. human brain suspension (0.6 Gm. per 10 ml. saline)

[4] PTA deficient plasma was obtained through the kindness of Dr. Robert Rosenthal.

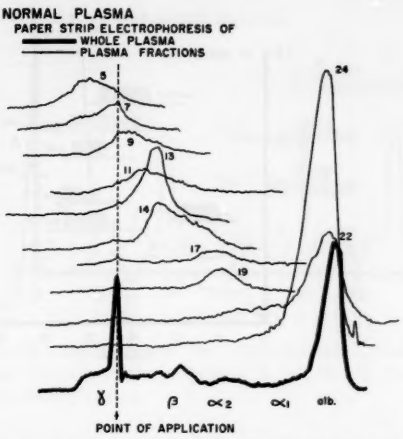

FIG. 3. ANALYTROL SCANS OF PAPER STRIP ELECTROPHORETIC PATTERNS OF FRACTIONS FROM CURTAIN ELECTROPHORESIS OF NORMAL PLASMA

in about 5 ml. saline, centrifuge at 20,000 rpm for 15 minutes, discard supernatant. Add 0.5 ml. $BaSO_4$ plasma eluate, 0.5 ml. imidazole buffer, 3.3 ml. saline and 0.5 ml. 0.1 M calcium chloride. After 15 minutes incubation add 0.5 ml. 0.1 M sodium oxalate and recentrifuge at 20,000 rpm for 15 minutes. Discard sediment. The $BaSO_4$ eluate was prepared by mixing 100 mg. $BaSO_4$ per ml. human plasma, sedimenting and washing the $BaSO_4$ and eluting prothrombin (plus other proteins) with one-eighth volume of 0.2 M sodium citrate.

Assay of fibrinolytic factors. Preliminary and simplified assays for components of the fibrinolytic enzyme system were undertaken.

1. Fibrinolysin. Mix 0.1 ml. fraction plus 0.1 ml. saline plus 0.1 ml. 1 per cent bovine fibrinogen[5] plus 0.1 ml. bovine thrombin[6] (20 units per ml.).

2. Proactivator or profibrinolysin. Mix 0.1 ml. fraction plus 0.1 ml. streptokinase[7] (200 units per ml.) plus 0.1 ml. bovine thrombin plus 0.1 bovine fibrinogen.

3. Profibrinolysin. Same as *2* but substitute human for bovine thrombin.

4. Antifibrinolysin. Mix 0.1 ml. fraction plus 0.1 ml. active dog fibrinolysin (15); incubate 60 minutes at 37° C., plus 0.1 ml. bovine thrombin plus 0.1 ml. bovine fibrinogen.

Materials

Early experiments were attended by numerous technical difficulties and are excluded from the series. Satisfactory normal experiments have included 24 on citrated plasma, 2 on serum, 3 on $BaSO_4$ treated oxalated plasma,

[5] The Armour Laboratories.

[6] Thrombin, Topical, supplied through the kindness of Parke, Davis and Co.

[7] Varidase®, supplied through the kindness of Lederle Laboratories Division, American Cyanamid Company.

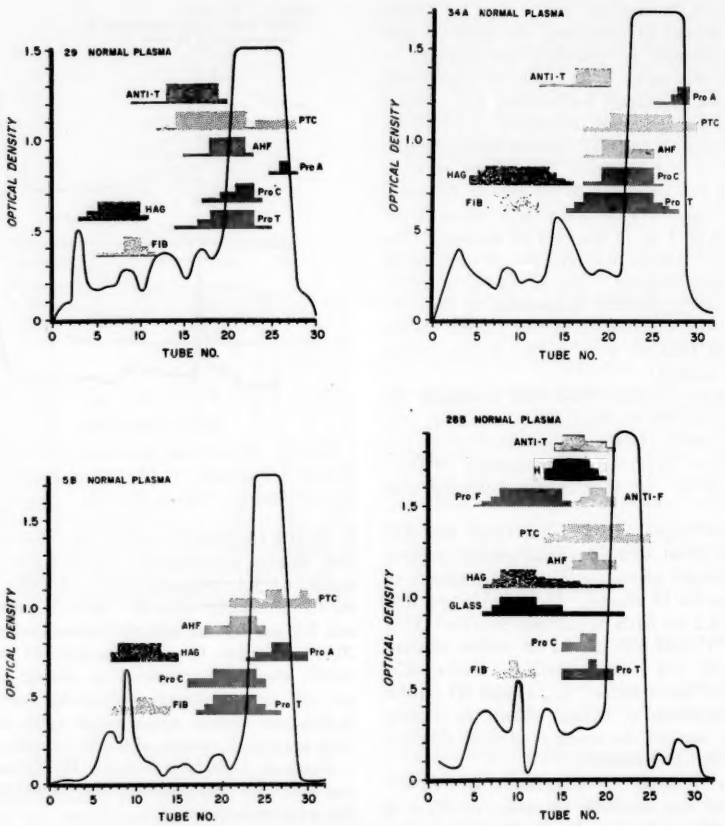

FIG. 4. DISTRIBUTION OF COAGULATION PROTEINS AFTER CURTAIN ELECTROPHORESIS OF NORMAL PLASMA

2 on $BaSO_4$ treated serum and 7 on various chemically prepared fractions.

$BaSO_4$ eluate. 100 ml. normal oxalated plasma was mixed with 10 Gm. $BaSO_4$ (Baker) for 10 minutes at room temperature; the mixture centrifuged 10 minutes at 4500 rpm; the sedimented $BaSO_4$ washed twice with 0.85 per cent NaCl and twice with distilled water, remixing and recentrifuging each time; and finally eluted by mixing with 15 ml. 0.2 M sodium citrate and centrifuging.

Ammonium sulfate fractions. $BaSO_4$ treated plasma (as prepared above) was fractionated by addition of saturated ammonium sulfate solution (room temperature). The precipitates, appearing after 28 per cent saturation and after sufficient additional ammonium sulfate solution was added to bring the concentration to 35 per cent, were collected by centrifugation and each was dissolved in one-fifth the plasma volume of distilled water. The supernatant was dialyzed alternately in distilled water and 25 per cent PVP (polyvinylpyrrolidone) until the volume and ionic concentration (as estimated by specific resistance measurements) returned to that of the original plasma. All fractions were dialyzed against barbital buffer before electrophoresis.

Fibrinogen. A human plasma fraction, Fibrinitol®, was provided through the courtesy of Ortho Research Institute.

RESULTS

Normal plasma. Comparison of curtain with paper strip electrophoresis

Samples from each tube from a number of curtains were analyzed by paper strip electrophoresis to determine mobility and homogeneity of the protein content. Figure 3 shows the results of a typical experiment. Commonly, tubes 1 to 13 contained γ globulin; 14 to 15, β globulin; 17 to 20,

α_2 globulin. α_1 globulin and albumin appeared in the large peak usually found between tubes 20 to 30. Not infrequently either one or two "pre-albumin" peaks were found in tube areas 30 to 32.

Distribution of coagulation factors

Figure 4 illustrates the distribution of coagulation factors among the various fractions in four runs on different normal plasmas. Fibrinogen, Hageman factor, "glass factor" and profibrinolysin were found in the γ area; heparin cofactor and antithrombin in the β to α_2 areas; prothrombin, proconvertin, AHF, PTC and antifibrinolysin in the α_2 to α_1 areas and proaccelerin in the albumin area.

Recovery of coagulation factors

Total recovery of fibrinogen, prothrombin, proconvertin, and proaccelerin was estimated in a number of experiments. Fibrinogen recovery was used as a criteria of a satisfactory run and if the total amount of fibrinogen found in all of the tubes was less than 20 per cent of that present in the volume of plasma electrophoresed, the tubes were discarded. Fibrinogen recovery varied from 20 to 106 per cent; prothrombin from 46 to 110 per cent; proconvertin from 26 to 80 per cent and proaccelerin from 0 to 28 per cent.

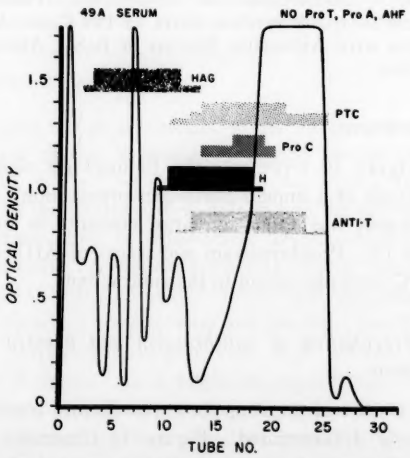

FIG. 5. DISTRIBUTION OF COAGULATION PROTEINS AFTER CURTAIN ELECTROPHORESIS OF NORMAL SERUM

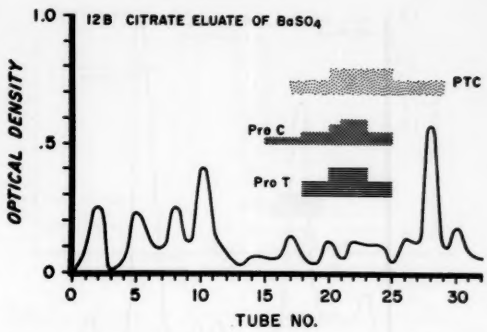

FIG. 6. DISTRIBUTION OF COAGULATION PROTEINS AFTER CURTAIN ELECTROPHORESIS OF THE $BaSO_4$ ELUATE OF NORMAL PLASMA

Normal serum

Figure 5 illustrates the results in a typical normal serum run. The protein concentration per tube was high and is reflected in the peaks found in tubes 2 to 13. As expected no fibrinogen was found. In this run (49A) prothrombin, proaccelerin and AHF were not detectable in the fractions although the whole serum contained 10 per cent AHF and 1 per cent prothrombin as compared to fresh plasma. In other serum runs traces of prothrombin have been found. Hageman factor, PTC and proconvertin appeared in their usual places. Heparin cofactor activity could be clearly differentiated from antithrombin activity although the two overlapped in a number of tubes.

$BaSO_4$ plasma

Three experiments on $BaSO_4$ adsorbed plasma showed no prothrombin, proconvertin or PTC, but the other coagulation proteins in their usual places. In one run, fractions were assayed for Hageman factor, glass factor and PTA. The three activities were found in the same fractions.

Plasma fractions

Certain plasma fractions prepared by chemical means were further studied and subfractionated by curtain electrophoresis.

$BaSO_4$ eluate

Tests on the whole fraction showed the presence of prothrombin, proconvertin, and PTC and

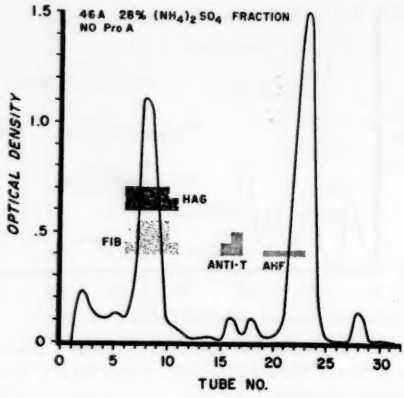

FIG. 7. DISTRIBUTION OF COAGULATION PROTEINS FOUND IN A 28 PER CENT AMMONIUM SULFATE FRACTION OF $BaSO_4$ ADSORBED PLASMA

these same materials were present in the electrophoretically prepared subfractions (Figure 6). As would be anticipated from their appearance in the same areas in plasma, no clear separation of these three components was achieved at this pH.

Ammonium sulfate fractions

Figures 7, 8 and 9 illustrate the results on continuous flow electrophoresis. The 28 per cent fraction appears to contain primarily γ globulin with a late peak in the albumin area. Hageman factor, fibrinogen, antithrombin and a trace of AHF were found. The 35 per cent fraction showed three distinct peaks, clearly visible on the stained curtain as well as in the protein pattern. Again, Hageman factor, fibrinogen, AHF and antithrombin were found. The supernatent from the 35 per cent fraction appears to contain primarily albumin, with smaller amounts of other components. The only coagulation factor found was the Hageman factor, present as usual in the γ area. None of the subfractions contained appreciable amounts of proaccelerin, which presumably deteriorated during the procedures.

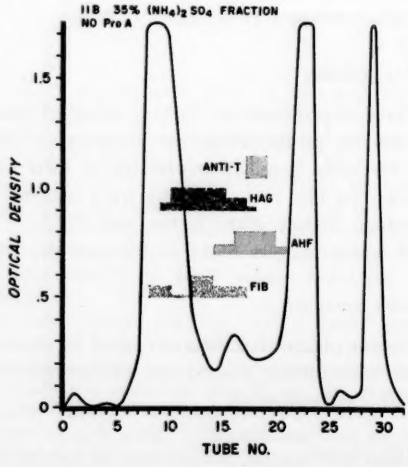

FIG. 8. DISTRIBUTION OF COAGULATION PROTEINS FOUND IN A 35 PER CENT AMMONIUM SULFATE FRACTION OF $BaSO_4$ ADSORBED PLASMA

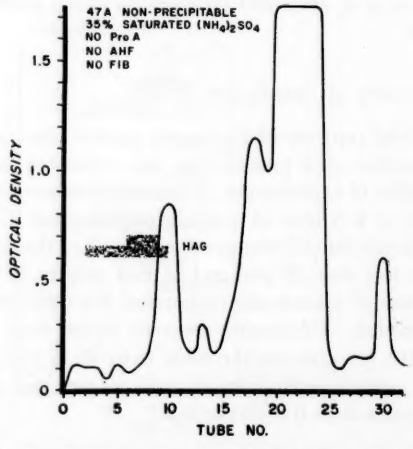

FIG. 9. DISTRIBUTION OF COAGULATION PROTEINS FOUND IN THE SUPERNATE AFTER 35 PER CENT SATURATION WITH AMMONIUM SULFATE OF $BaSO_4$ ADSORBED PLASMA

Fibrinogen

Figure 10 represents the findings on electrophoresis of a human fibrinogen preparation. Fibrinogen was found in large amounts in tubes 7 to 12. Profibrinolysin and traces of AHF and PTC were also found in the subfractions.

Differentiation of antithrombin and heparin cofactor

In most of the runs these two factors could be clearly differentiated. Figure 11 illustrates the detailed tests in a run on $BaSO_4$ treated plasma (29B). The control tests, in which each fraction

plus saline plus fibrinogen was clotted with thrombin, showed approximately the same clotting time in all tubes. If, instead, the fractions were tested for antithrombin by first incubating with thrombin and then clotting with added fibrinogen, tubes 19 to 28 could markedly inhibit the thrombin. However, if each fraction was mixed with heparin and fibrinogen, then clotted with thrombin, tubes 17 to 24 showed marked heparin potentiating effect. Thus, although there was an overlap, at least two tubes on each end showed primarily a single activity.

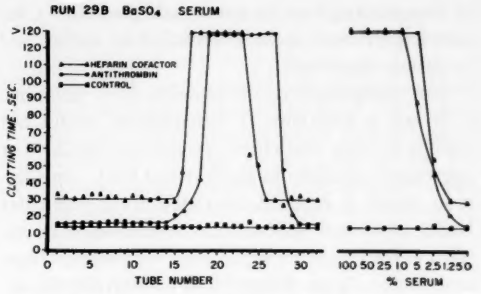

FIG. 11. ASSAY OF HEPARIN COFACTOR AND ANTITHROMBIN

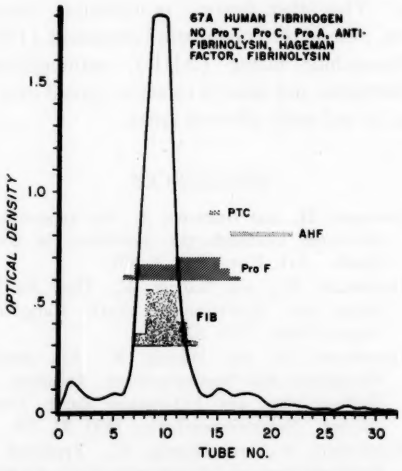

FIG. 10. DISTRIBUTION OF COAGULATION PROTEINS FROM A PLASMA FRACTION HIGH IN FIBRINOGEN CONTENT

Fibrinolytic enzyme system

Figure 12 shows details of the tests for components of this system in a typical plasma run (66A). The test for fibrinolysin shows that none of the fractions were able to lyse the clot and therefore must be considered devoid of active fibrinolysin. Antifibrinolysin was clearly demonstrated in tubes 15 to 25. Profibrinolysin, tested by mixture of streptokinase and fractions in a bovine thrombin-fibrinogen clot, was present in tubes 5 to 16. As it might be argued that this test was actually measuring the presence of a streptokinase sensitive activator system (16) in the human fractions which, in turn, activated bovine profibrinolysin present in the fibrinogen or thrombin, an additional test was run in which human thrombin was substituted for the bovine thrombin. This human thrombin should provide a constant supply of the activator system so that the profibrinolysin in the fibrinogen of all tubes would be activated. The results (not illustrated) showed lysis times in tubes 5 to 16 identical to those found with bovine thrombin. All of the other tubes did show a slow lysis (40 to 60 minutes) indicating activation of a trace of contaminating profibrinolysin present in either the thrombin or the fibrinogen.

DISCUSSION

These experiments demonstrate the usefulness of the continuous flow electrophoretic technique in fractionating plasma proteins. Although in this early stage in the use of this technique, complete separation of the various clotting factors has not been achieved, yet their recovery in apparently undenatured form and in volume adequate for many studies suggests that the general principles

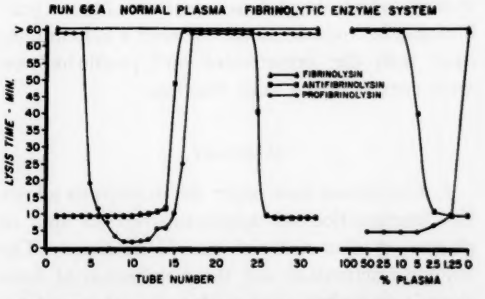

FIG. 12. ASSAY OF FIBRINOLYSIN, PROFIBRINOLYSIN AND ANTIFIBRINOLYSIN

of flow-electrophoresis are worth pursuit. Current experiments include variation in buffer and in curtain material.

The categorization of proteins into albumins, α, β and γ globulins, is descriptively useful but applies to only one characteristic—molecular (or aggregate) mobility in an electrical field. In common usage it is even restricted to a particular buffer system. When such terminology is applied to the coagulation proteins, fibrinogen, Hageman factor, "glass factor" and profibrinolysin appear to be γ globulins, proaccelerin an albumin, and the rest mixtures of α_1, α_2, some albumin and some β. In these preliminary stages, such spreading does not necessarily indicate that a given biological activity is associated with protein molecules of different size, configuration or charge.

Certain interesting and useful observations were made. The distribution of glass factor activity coincided with Hageman factor, suggesting that these may be identical. The identification of glass factor with Hageman factor has been previously suggested by Ratnoff and Rosenblum (17). It was possible to assay for PTA activity in only one run. In this experiment PTA activity appeared to coincide with Hageman factor and with "glass factor" activities. This close association suggests that these apparently different disorders (Hageman trait and PTA deficiency) may be closely related. It is hoped to pursue this matter by fractionation of both types of deficient plasmas. Activities of heparin cofactor and antithrombin were shown to be distinct.

Preliminary tests for the fibrinolytic enzyme system showed the presence of profibrinolysin and antifibrinolysin. No real indication of the presence of a proactivator-activator mechanism (16) through which streptokinase activates human profibrinolysin could be found. If such a system does exist both the proactivator and profibrinolysin must coexist in the same fractions.

SUMMARY

1. Continuous flow paper electrophoresis allows the fractionation of approximately 15 ml. of plasma or other material into 32 fractions. The volume fractionated and the distribution of fractions is dependent upon such technical considerations as applied voltage, buffer flow, wick flow and plasma flow. All experiments in this study employed barbital buffer pH 8.6, ionic strength 0.02, and special cooling technique.

2. Content of coagulation proteins in each fraction was determined by measuring their various biological activities. High recovery of such activities suggested that little or no denaturation had occurred.

3. Fibrinogen, profibrinolysin, Hageman factor, plasma thromboplastin antecedent (PTA) and "glass factor" appeared in the γ globulin area. The latter three are similar in their fraction distribution. Proaccelerin appeared in the albumin area. The other factors, prothrombin, proconvertin, plasma thromboplastin component (PTC), antihemophilic factor (AHF), antifibrinolysin, antithrombin and heparin cofactor, spread over the β, α_2, α_1 and early albumin areas.

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